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T-lymphocyte/monocyte interactions in relation to inflammatory joint diseases

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T-lymphocyte/monocyte interactions in relation to inflammatory joint diseases

Submitted by

Ahmed Khalifa Ali Al-Shukaili

For the degree of Doctor of Philosophy

of the University of Bath

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease affecting the synovial membranes of multiple joints. Elevated levels of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) have been detected in RA and are considered to be key mediators of the disease process. Hypoxia up-regulates TNF-α and IL-1β; therefore, considerable research interest has been focused on the biological consequences of the hypoxic nature of the rheumatoid synovium. A hydrogen peroxide tolerant Jurkat cell line (HJ16) was generated to study the mechanisms of cytokine production under oxidative stress. HJ16 and normal Jurkat (J16) cells were co-cultured with a monocyte cell line (THP-1) or peripheral blood mononuclear cells (PBMCs). IL-1β and IL-1 receptor antagonist (IL-1ra) production by THP-1 cells or PBMCs was measured.

HJ16 cells exhibited different characteristics to J16 cells. HJ16 cells were slightly activated at resting conditions, manifested by low expression of CD69. Also HJ16 cells have higher level of glutathione (GSH) compared to J16 cells. HJ16 cells were more sensitive to PHA-L than J16 cells, manifested by higher expression of CD2 and low proliferation rate after activation. In co-culture, activated unfixed HJ16 cells stimulated more IL-1β production by both PBMCs and THP-1 cells, although they exhibited reduced calcium signalling. Activated fixed T cells (J16 and HJ16 cells) failed to stimulate (to significant levels) both IL-1β and IL-1ra production by PBMCs, but significantly stimulated IL-1ra production by THP-1 cells, compared to unfixed activated T cells. Activated fixed HJ16 cells induced slightly higher levels of IL-1ra production by THP-1 cells. Depletion of GSH in J16 and HJ16 cells augmented the stimulation of IL-1β and IL-1ra production by THP-1 cells, but reduced calcium signalling and proliferation. Enhancement of GSH levels in J16 or HJ16 cells had no effect on the stimulation of IL-1β or IL-1ra production by THP-1 cells.

Chronic exposure to oxidative stress such as H_2O_2, and decreased antioxidants (GSH) in T cells have altered their characteristics, and enhanced their stimulation of proinflammatory cytokine production by monocytes-macrophages.

CONTENTS

Abbreviations

1,15 $(OH)_2$ D_3	1,25-dihydroxyvitamin D3
APC	Antigen presenting cells
Apo-1	Apolipoprotein A-I
ATP	Adenosine triphosphate
BIM	bisindolylmaleimide
BSA	Bovine serum albumin
BSO	Buthionine-DL-sulphoximine
COX	Cyclooxygenase
CSF	Colony stimulating factor
DEM	Diethyl maleat
DMSO	Dimethyl sulphoxide
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (b-amino-ethyl ether) N, N, N, tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENA-1	Epithelial neutrophils antigen-1
ERK	Extracellular signal-regulated kinase
FACS	Extracellular
FcR	Fc receptor
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSH	Glutathione (reduced)
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
H2O2	Hydrogen peroxide
ICE	Interleukin-1 converting enzyme (caspase-1)
IFNγ	Interferon gamma
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IL-1RI/IL-1RII	Interleukin-1 receptor type I and II
IP3	Inositol-1, 4, 5 triphosphate
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
NAC	N-acetylcysteine
NADH	Nicotinamide adenine dinucleotide (reduced)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
Na, K-ATPase	Sodium/potassium adenosine triphosphatase
PBMC	Peripheral blood mononuclear cell
PDBu	Phorbol dibutyrate
PGE_2	Prostaglandin E_2
PHA	Phytohaemagglutinin
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase

PKC	Protein kinase C
PMA	Phorbol myristate acetate
PMS	Phenazine methosulfate
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROS	Reactive oxygen species
TCA	Trichloroacetic acid
TGFβ	Transforming growth factor beta
Th cells	T helper cells
TNF-α	Tumour necrosis factor alpha
Tr cells	Regulatory T cells
TRX	Thioredoxin
XTT	Sodium 3'-1[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate

CHAPTER 1

INTRODUCTION

Chapter 1 Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disorder that affects approximately 1% of the population, in a female/male ratio of 3/1. The disease can occur at any age, but it is most common among those aged 40-70 years. Despite many years of study, the aetiology of RA is still undefined. However, with increased understanding of the immune system the pathogenesis of RA has become clearer. A large bulk of data suggests that T lymphocytes and macrophages play a critical role in the initiation and perpetuation of synovial inflammation (McCarty, 1989; Sewell & Trentham, 1993; Snowden & Kay, 1995; Panayi, 1999; Weyand, 2000).

Recently, the cytokine profile of T helper cells has been associated with the disease, the cytokine repertoire of inflamed synovia is categorised as that of T helper 1 response. Moreover, in RA elevated levels of proinflammatory or inflammatory cytokines such as TNF-α and IL-1β have been detected. Hypoxia up-regulates TNF-α and IL-1; therefore, considerable research interest has been focused on the biological consequences of the hypoxic nature of the rheumatoid synovium. Hypoxia might underlie the functional polarization of the T cells and cytokine production, and thus may contribute to the progression and persistence of the disease (reviewed by Dayer & Fenner, 1992; Feldman et al, 1996; Miossec & Berg, 1997; Bodamyali et al, 1998).

In addition, cellular antioxidants such as glutathione (GSH) regulate lymphocyte function. GSH is known to mediate lymphocyte growth and activation as well as their responsiveness to cytokines, (Suthanthiran et al, 1990; Staal et al, 1994; Maurice et al, 1997; Verhasselt et al, 1999; Hehner et al, 2000; Sido et al, 2000; Roozendaal et al, 2002; Monick et al, 2003). For example, treatment with buthionine sulfoximine

(BSO) to deplete GSH and N-acetyl-L-cysteine (NAC) to enhance GSH, caused a decrease and increase in lymphocyte proliferation respectively (Maurice et al, 1997). Moreover, NAC-treatment resulted in upregulation of IL-4 (Th2 cytokine) and down regulation of IFN-γ (Th1 cytokine) production, thus GSH may mediate T cell phenotype (Monick et al, 2003).

Direct cell contact with T lymphocytes is a potent mechanism, which induces the production of proinflammatory cytokines by monocytes-macrophages. This interaction represents an important mechanism for initiation of the inflammation and mediates tissue destruction. Through this interaction, mononuclear phagocytes are capable of mediating tissue destruction directly by secreting matrix metalloproteinases (MMPs) or indirectly by releasing cytokines such as IL-1β and TNF-α, which in turn induce the production of MMPs by connective tissue cells. *In vitro*, T cell-induced contact-dependent activation of macrophages is a potent mechanism to induce IL-1β, TNF-α (Dayer et al, 1985; Chizzolini et al, 1997; Vey et al, 1997) and MMPs (Lacraz et al, 1994; reviewed by Burger & Dayer, 2002).

The aim of this research is to determine whether oxidative stress or redox regulation alters the behaviour of the T cells and their interactions with monocytes-macrophages in relation to inflammatory joint disease.

1.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic recurrent, systemic inflammatory disease primarily involving the synovial membranes of multiple joints, followed by cartilage and bone destruction, which eventually lead to joint deformities and loss of articular functions. The clinical course of the disorder is extremely variable, ranging from mild, self-limiting arthritis to rapidly progressive multi-system inflammation with profound morbidity and mortality.

The predominant symptoms of RA are pain, stiffness, and swelling of peripheral joints. These symptoms are accompanied by signs of articular inflammation, including swelling, warmth, erythema, and tenderness on palpation. At the onset of the disease small joints of the hand and feet are involved. Large joints such as knees, hips, elbows, ankles and shoulders commonly become involved later in the course of the disease. In the most severe cases, symptoms can include lesions of tendons, skeletal muscle, peripheral nerves and arteries (McCarty, 1989; Sewell & Trentham, 1993; Snowden & Kay, 1995).

1.1.1 Aetiology of RA

The cause of RA remains obscure. Over the years, a number of potential etiologic agents have been proposed. Some of these agents are illustrated in Table 1. Although many of these suggested pathogens could cause RA, to date no etiologic agent has been conclusively confirmed for RA (Venables, 1989; Hyrich & Inman, 2001).

Table 1 Proposed etiologic agents for RA

Bacteria	*Mycobacteriae*
	Escherichia coli
	Proteus mirabilis
	Streptococcus Group A
	Staphylococcus
	Mycoplasma arthritidis
	Other
Viruses	*Epstein-Barr virus*
	Cytomegalovirus
	Rubella virus
	Hepatitis B
	HTLV-1
	Other

The mechanism by which any microorganisms may cause RA also remains unclear. However, there are some proposed mechanisms such as (1) the direct infection into the synovium or retention of microbial products may trigger the chronic inflammatory response, (2) the infection or the host response may alter articular structures and they become immunogenic (Tarkowaski et al, 1989), (3) the etiologic agent might prime the host to a cross- reactive determinant expressed in the joint as a result of molecular mimicry (Winchester et al, 1988) and (4) superantigens might play a role in initiation of the disease. Superantigens are proteins that can bind to major histocompatibility complex (MHC) class II and certain Vβ segments of the heterodimeric T cell receptor. Because of this binding, superantigens are potent stimulators of T cells (Choi et al, 1990).

Auto-antigens may also be involved in the initiation of RA, including the cartilage proteoglycan component aggrecan, human cartilage glycoprotein (HCgp-39) and p78. Aggrecan has been found in the synovial fluid of RA patients (Lohmander et al, 1993; Boots et al, 1997), can induce inflammatory arthritis in murine models (Glant et al,

1987), and exhibits multiple sites capable of binding to peptide motifs for HLA-DR1 and HLA-DR4 (Boots et al, 1997). HCgp-39 was detected in PBMCs and synovial samples in RA patients but not in normal subjects (Baeten et al, 2000; reviewed by Corrigall & Panayi, 2002).

There is a convincing association between the expression of certain class II MHC gene products (Human Leukocyte Antigen (HLA)-DR) and the development and severity of RA. A large body of evidence has shown that, there is a strong association between RA and the presence of DR4 (Gregersen et al, 1987; Scott, 2000). In addition, there is association between MHC haplotype and disease severity of RA. For example, patients with homozygous DR4 tend to have more extra-articular manifestations as well as more destructive joint involvement than those with only one allele. However, those patients with one allele, in turn tend to have more severe disease than those patients with other MHC haplotypes (Gregersen et al, 1987; Wordsworth et al, 1989; Ollier & MacGregor, 1995; Singeal et al, 1999; Patil et al, 2001).

The HLA–DR molecule is composed of an invariant α-chain and a highly polymorphic β–chain. The antigen-binding cleft formed by α and β chains of the molecules contains the amino acid sequence known as the shared epitope in the β1 chain of DR4 and also on β1 chain encoded by some other DR genes, such as DR1. The epitope common to those HLA-DR molecules is found from amino acid 70-74. The shared epitope may serve as the binding site for an arthritogenic peptide or may itself be the autoantigen that activates T cells (Gregersen et al, 1987; Singeal et al, 1999, Smith & Haynes et al, 2002).

Moreover, the MHC class II role may predispose to disease by positively selecting for auto reactive clones of T cells or by negatively selecting against immuno-regulatory clones (reviewed by Kinne et al; 1997, Smith & Haynes et al, 2002).

1.1.2 Pathogenesis of RA

The initial stage in rheumatoid synovial inflammation involves some replication of cells in the synovial membrane to form a multi-cellular layer, which thickens primarily due to the simultaneous recruitment to this area of large numbers of macrophages, although T cells ($CD4^+$, $CD45RO^+$) are also present (Sewell & Trentham, 1993).

In the chronic phase of RA, infiltration of lymphocytes and macrophages becomes more abundant. Moreover, the chronic synovial inflammation in RA results in the formation of the pannus. This highly vascularized granulation tissue is composed of a variety of cell types, including not only lymphocytes and macrophages but also fibroblast synoviocytes and mast cells. Pannus is found at the marginal areas of diarthrodial joints, where it impinges upon articular cartilage and subchondral bone (Allard et al, 1987; Sewell & Trentham, 1993; Palmer, 1995; Tak et al, 1997).

The role of T cells and macrophages in RA is discussed in more detail in sections 1.4 and 1.5. However, it is likely that mature T cells ($CD4^+/CD45RO^+$) are capable of triggering several factors responsible for rheumatoid symptoms, including cytokine release and immunoglobulin (Ig) production by B cells (Panayi et al, 1992). IgM (the most prominent isotype) and IgG rheumatoid factors (RFs), together with anti-type-II-collagen activity and antibodies to collagen types V, VI and IX have been detected in

the synovium of RA patients (Tarkowaski et al, 1989). The binding of such immune complexes to cartilage could exacerbate the immune response by activating complement and triggering further cellular inflammatory response (Sewell & Trentham, 1993).

The maintenance of the integrity of cartilage and other extracellular matrix (ECM) tissue is a dynamic process. In the normal state, the synthesis of ECM components by chondrocytes and fibroblasts is counterbalanced by a specific degradation of these molecules. Degradation is mediated by a family of proteolytic enzymes (Matrix Metalloproteinases, MMPs), produced by both macrophages and activated fibroblasts in response to proinflammatory cytokines such as interleukin-1-β (IL-1β) and tumour necrosis factor-α (TNF-α). Important enzymes in this category include collagenase (MMP-1), gelatinase (MMP-2) and stromelysin-1 (MMP-3) (Manicourt et al, 1995; Honda et al, 2001; Vincenti et al, 2002) These enzymes are secreted as proenzymes, which are later activated by proteolytic activity of other enzymes, such as trypsin and plasmin, these activated enzymes are regulated by inhibitory proteins such as tissue inhibitor of metalloproteinases (TIMPs). These are also released by macrophages and fibroblasts forming the pannus (Nemoto et al, 1996). In addition, there are other proteases that probably play an important role in tissue damage in RA include elastase and cathepsin G, secreted by macrophages and other inflammatory cells (Wright et al, 1991; Mauviel, 1993; Vincenti et al, 1994; Feldmann et al, 1996).

Cytokines play a crucial role in mediating tissue damage (see section 1.3). IL-1, IL-6 and TNF-α may directly activate osteoclasts, stimulate cyclooxygenase-2 (COX-2) resulting in increased prostaglandin E_2 (PGE_2) production and may cause ECM

degradation via their ability to stimulate collagenase and stromelysin production by synoviocytes and chondrocytes (reviewed by Dayer & Fenner, 1992, Masferrer et al, 1994; van de Loo et al, 1995; Feldman et al, 1996; Feldman & Maini, 1999).

The increase in intercellular adhesion molecules-1 (ICAM-1) expression, which accompanies the thickening of the pannus, is also enhanced by the action of IL-1β, IL-6, and IFN-γ (Dayer & Fenner, 1992; Koch et al, 1992; Arias-Negrete et al, 1995; Lee et al, 1995; Van de Loo et al, 1995; Burmester et al, 1997). The pathogenesis of RA is illustrated diagrammatically in Figure 1.

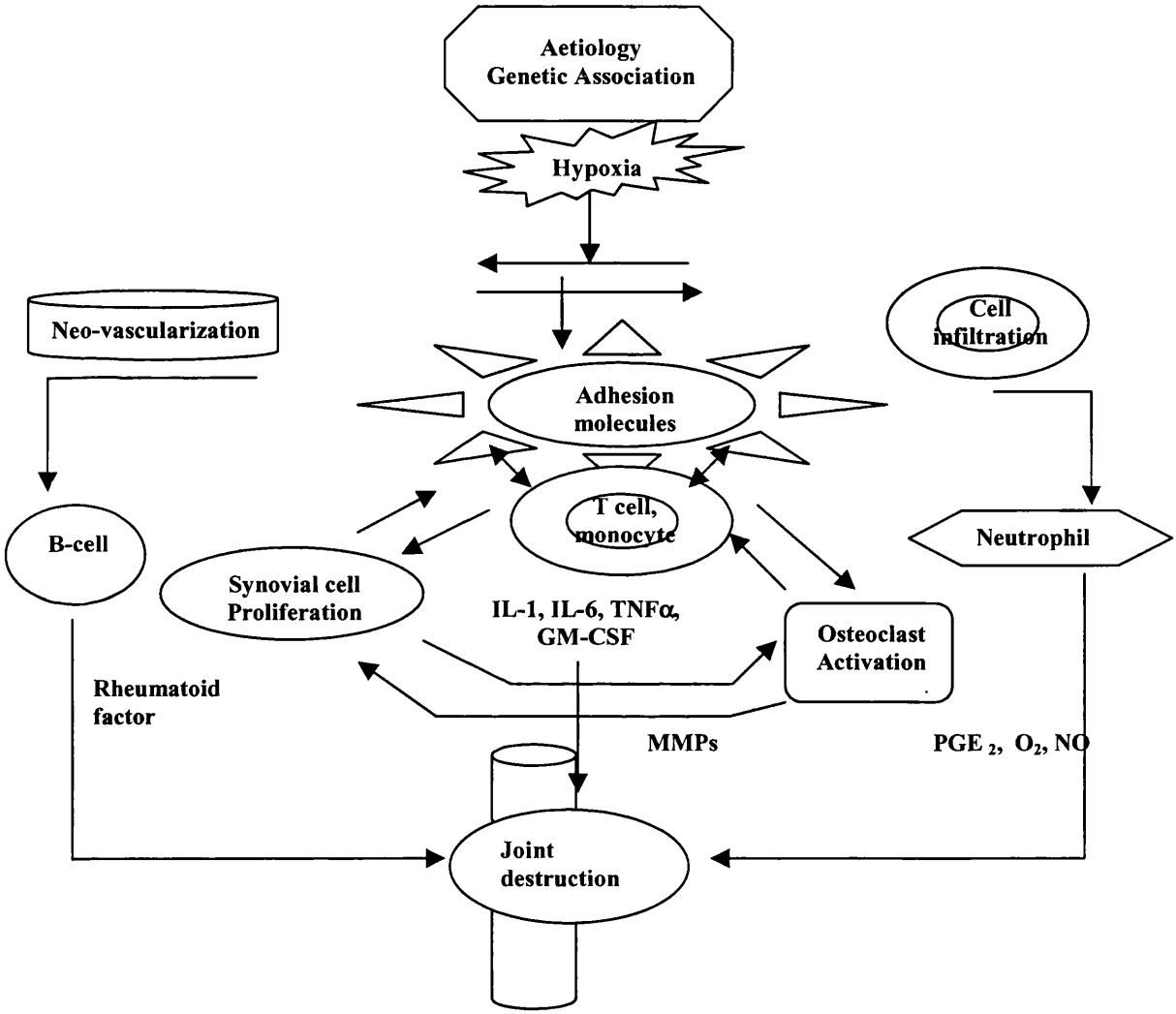


Figure 1: **Pathogenic pathways of joint destruction in RA patients**. Rheumatoid arthritis is characterized by neo-vascularization and mononuclear cell infiltration. Infiltrating mononuclear and synovial cells are activated and contribute to the perpetuation of rheumatoid inflammation mediated by the cytokine network. Eventually, joints are destructed by various enzymes produced by theses activated cells.

1.2 Hypoxia, oxidative stress and RA

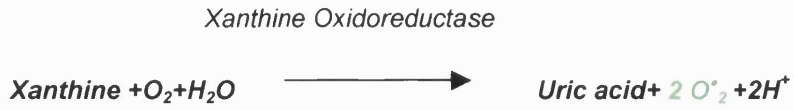
The rheumatoid synovial cavity has a positive intra-articular pressure, in contrast to a normal joint, which has negative pressure. In RA synovium, the cavity pressure is raised and upon movement this pressure exceeds the capillary perfusion pressure, causing collapse of the blood vessels. This leads to the production of multiple episodes of hypoxic reperfusion injury, which consequently generates reactive oxygen species (ROS) (Mapp et al, 1995).

The earliest view of the redox concept is that of the addition of oxygen molecules (oxidation) to from an oxidant, or removal of oxygen (reduction) to form a reductant. Alternative approach of redox concept is to describe the oxidation as the loss of hydrogen, and reduction as the gaining of hydrogen. Furthermore, another alternative view is to describe oxidation as the losing of electrons and reduction as gaining of electrons (Haddad et al, 2002b).

ROS such H_2O_2, superoxide ($O_2^{\cdot-}$) and the hydroxyl radical ($OH^{\cdot}$), are generated in cells by several pathways: cellular energy metabolism to generate ATP in mitochondria involves the electron transport reaction in which O_2 accepts electrons and H^+ to yield water, in this process a single electron may leak out resulting in the production of $O_2^{\cdot-}$. Another site of electron transport is the endoplasmic reticulum, where $O_2^{\cdot-}$ is released from NADPH cytochrome P450 reductase (reviewed by Kamata & Hirata, 1999). $O_2^{\cdot-}$ is also generated by hypoxanthine/xanthine oxidase (Figure 2) (Mapp et al, 1995; reviewed by Kamata & Hirata, 1999).

Figure 2: Generation of ROS:

1.Generation of Superoxide anion.



2. Generation of Ferrous irons

3. Generation of H_2O_2: Trauma caused by movement of the joint leads to H_2O_2 release by neutrophils: $2O^{-}_2$+ $2H^+$

4.Release of hydroxyl radicals:

There are several pieces of evidence that indicate the hypoxic nature of RA synovium, including: direct measurement of O_2 in synovium revealed low O_2 and raised levels of CO_2, decreased synovial pH, raised lactate, 3-D-hydroxybutyrate, acetate levels, and ketone body formation. Increased enzyme activity such as glyceraldehyde-3 phosphate dehydrogenase, glucose 6-phosphate dehydrogenase and lactate dehydrogenase (major enzymes of the glycolytic pathway) confirmed the hypoxic nature of the RA synovium. Moreover, increased intracellular but decreased extracellular sulphydryl content within the inflamed synovium is a strong indication of redox imbalance (reviewed by Bodamyali et al, 1998).

The hydroxyl radicals are very highly reactive, attacking a wide range of targets including hyaluronic acid in the synovial fluid, causing loss of lubricant properties and consequently mechanical damage to the joint (Blake et al, 1989; Schett et al, 2001). The hydroxyl radicals break single and double stranded DNA. Moreover, ROS activate nuclear factor κB (NF-κB), a transcription factor that mediates expression of certain cytokines genes such as TNF-α and IL-1 (Tacchini et al, 1995; Chandel et al, 2000). Hydroxyl radicals attack amino acids, and thus may be involved in the formation of rheumatoid factors (IgG, IgM and IgA). Proteins modified by hydroxyl radicals, could induce autoimmune responses and release of autoantibodies by B cells (Blake et al, 1989; Stevens et al, 1991; Fairburn et al, 1993; Mapp et al, 1995; Bodamyali et al, 1998).

Oxidative stress also has an effect on T cells; it has been observed that addition of an antioxidant resulted in reduction of T cell proliferation, IL-2R expression and IL-2 production (Chandhri et al, 1988). Hypoxia transcriptionally upregulates TNF-α and IL-1 (Ghezzi et al, 1991; Naldini et al, 2001) but down regulates IL-2, therefore hypoxia might underlie the functional polarization of Th1/Th2 cells and so favour proinflammatory cytokines such as IL-1 and TNF-α (Blake et al, 1989; Stevens et al, 1991; Fairburn et al, 1993; Mapp et al, 1995; Bodamyali et al, 1998). Moreover, low concentration of H_2O_2 caused activation of T cells (reviewed by Ginne-Pease & Whisler, 1998), and down-regulation of CD3 expression (Kono et al, 1996).

In addition, PKC activity is susceptible to oxidative stress or H_2O_2 (Whisler et al, 1995; reviewed by Gopalakrishna & Jaken, 2000). MAPKs, PTK, PLCγ are also activated by H_2O_2 and oxidative stress (Goldstone et al, 1997; Kamata & Hirata,

1999; Devadas et al, 2002). The transcription factor NF-κB mediates the LPS-induced release of cytokine gene transcription. One common mechanism for the activation of NF-κB is requirement for an increase in ROS. Exposure to H_2O_2 or hypoxia can rapidly activate NF-κB (Brennan & O'Neill, 1995; reviewed by Ginn-Pease & Whisler, 1998; Janssen-Heininger et al, 1999; Chandel et al, 2000).

As protection against oxidative stress, cells possess anti-oxidants that maintain the intracellular redox environment. Glutathione (GSH) is a major cellular anti-oxidant (see section 1.2.1) found in all eukaryotic cells. Cellular H_2O_2 is eliminated in the cytoplasm by GSH-peroxidase-catalysed reduction, with GSH as a substrate. As a result of this reaction, oxidised GSH (GSSG) is formed, and then the GSSG is restored to GSH by GSH reductase. Thioredoxin (TRX) is a small multifunctional cellular protein that is also capable of reducing some ROS including H_2O_2. Moreover, H_2O_2 can be eliminated by the enzyme catalase in the peroxisomes (reviewed by Mapp et al, 1995; Bodamyali et al, 1998; Kamata & Hirata 1999).

1.2.1 Glutathione and redox balance

Glutathione (L-γ-Glutamyl-L-cysteinylglycine, GSH) is a major intracellular non-protein sulphydryl anti-oxidant, and plays a central role in defending cells against free radicals and electrophiles and maintaining cellular oxidation-reduction (redox) equilibrium. The protective role of GSH consists of four components: 1) chemical reaction with intracellular targets (in most cases DNA); 2) enzymatic reduction of peroxides to prevents their decomposition; 3) enzymatic detoxification of electrophiles, as a coenzyme for formaldehyde dehydrogenase and glyoxylase and as

a substrate for the GSH S-transferase, and 4) maintenance of the redox state of cellular thiols. Most cell types can metabolise extracellular GSH by an ectoenzyme, γ-glutamyl transpeptidase (GGT), which transfers glutamate to amino acid acceptors. The rate of transport and the intracellular availability of cysteine appear to control the rate of GSH synthesis (Kavanagh et al, 1988 & 1994; Shrieve et al, 1988; Cook et al, 1991; Ublacker et al, 1991; van der Ven et al, 1994; reviewed by Anderson, 1998; Seres et al, 2000).

The balance between reduced and oxidized glutathione is an important mechanism for balancing the effect of reactive oxygen species and alkylating agents. Oxidized glutathione can modify selected proteins in a process referred to as S-glutathiolation and is used as a means of minimizing oxidative damage. These reversible disulfide bonds are an effective means of protecting modified proteins against irreversible oxidative damage. Redox signalling involves modification of cellular proteins, and it leads to a decrease in the cellular glutathione levels [glutathione depletion has an effect on gene expression] (Coan et al, 1992; Park et al, 1998).

On one hand, GSH depletion is associated with augmentation of an oxidative stress-mediated production of proinflammatory cytokines. It has been shown that pre-treatment with L-buthionine (S-R) sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, prior to exposure to LPS, augmented in a dose-dependent manner LPS-induced TNF-α and IL-6 biosynthesis in alveolar epithelial cells, an effect associated with the induction of intracellular accumulation of reactive oxygen species (ROS) (Haddad, 2001a/b), and reduced IL-12 production by human

alveolar macrophages (Dobashi et al, 2001). Moreover, BSO treatment of bronchial epithelial cells (BECs) facilitated TNF-α-induced mitogen-activated protein kinase (MAPK) p38 activation and RANTES production (Hashimoto et al, 2001). Pre-treatment of monocytes with maleic acid diethyl ester (DEM) to reduce GSH levels also resulted in a decrease in IL-12 generation (Utsugi et al, 2002).

On the other hand, antioxidant drugs can in turn act as modulators of the immune response. In particular, N-acetylcysteine (NAC), through interfering with or counteracting ROS-induced subcellular changes can modulate several cell functions. This drug in fact works as a free radical scavenger, as a GSH precursor via a thiol supplier activity, or as structural analogue of GSH, enriching the GSH pool in the cellular environment (reviewed by Zafarullah et al, 2003). NAC treatment induced a significant upregulation of IL-1β, IL-2, IL-15 (Viora et al, 2001), and IL-12 production (Dobashi et al, 2001; Viora et al, 2001). In addition, pre-treatment with NAC reduced LPS-mediated secretion of TNF-α and IL-6, incubation of cells with NAC induced intracellular accumulation of GSH (Haddad, 2001b). Moreover, NAC attenuated TNF-α-induced IL-8 production, and also attenuated TNF-α-induced activation of MAPK, p38 (Hashimoto et al, 2001). Furthermore, in contrast to BSO, which activates NF-κB, antioxidants such as NAC inhibit the activation of NF-κB and AP-1, and consequently block transcription of cytokine genes (reviewed by Ginn-Pease & Whisler, 1998; Chandel et al, 2000).

It is now well documented that oxidative stress triggers several intracellular pathways that lead to activation of certain transcription factors and expression of specific genes

as mentioned above. Signal transduction via MAPK is involved in this process. MAPKs include the c-Jun N-terminal kinases (JNKs), p38 kinases and extracellular signal–regulated kinases (ERK1/2). JNK and p38 are induced by stress responses and mediating responses to inflammatory cytokines (Hale et al, 1999; Rincon et al, 2000).

MAPK cascades are involved in inflammation and tissue destruction in RA. In particular JNK is highly activated in RA fibroblast-like synoviocytes and synovium (reviewed by Vincenti et al, 2001). MAPK specificity may exist with respect to MMP-1 and MMP-13 gene expression. Although all three MAPK pathways can contribute to MMP-1 expression in synovial fibroblasts, cytokine activation of MMP-13 in chondrocytes appears to be primarily p38-dependent, since it is blocked with the p38 kinase inhibitor SB203580 (reviewed by Vincenti et al, 2001). In addition, Han et al found that administration of SP600125 (JNK inhibitor) decreased the rat paw swelling in rat-adjuvant-induced arthritis, and near-complete inhibition of radiographic damage that was associated with decreased AP-1 activity and collagenase-3 gene expression (Han et al, 2001).

NAC attenuated TNF-α induced p38 MAPK activation and RANTES production and BSO facilitated TNF-α /LPS-induced p38 MAPK activation (Haddad, 2001b; Hashimoto at al, 2001). These observations indicate an association between the cellular reduction/oxidation (redox) state and p38 MAPK-mediated cytokine expression.

Lymphocyte function is particularly dependent on GSH levels. GSH is known to influence lymphocyte growth and activation as well as their responsiveness to

cytokines. RA synovial fluid T cells contain decreased GSH levels compared to cells isolated from peripheral blood of RA patients or healthy people (Maurice et al, 1998). However, synovial T cells show features of hyporesponsiveness. This hyporesponsiveness is reflected by low proliferative responses (Maurice et al, 1997, 1998), as well as low calcium responses upon stimulation (Carruthers et al, 1996; 2000). It has been shown that depletion of intracellular GSH levels in T lymphocytes through treatment with BSO results in hyporesponsiveness due to the abrogation of the proximal TCR-mediated signalling (Staal et al, 1994) and displacement of the adaptor protein Linker for Activation of T cells (LAT) from the plasma membrane (Gringhuis et al, 2000). This has been also observed in SF T cells in RA, may be due to subjection to chronic oxidative stress and decreased GSH levels in these cells (Gringhuis et al, 2000 & 2002).

Antigen presenting cells (APC), macrophages, dendritic cells and B cells are central to the development of either T helper 1 (Th1) or T helper 2 (Th2) immunity. GSH depletion in APC inhibited Th1-associated cytokine production (IFN-γ) (Peterson et al, 1998). Furthermore, decreased intracellular GSH levels in macrophages resulted in upregulation of the production IL-6 and IL-10 (critical effector cytokine of Th2), and down regulated IL-12 (Th1 cytokine), thus GSH plays an important role in the polarization of Th1/Th2 balance (Murata et al, 2002). However, increasing intracellular thiol pools with NAC in splenocytes resulted in upregulation of IL-4 production (Th2 cytokine) after anti-CD3 stimulation (Monick et al, 2003).

1.3 Cytokines and RA

Rheumatoid arthritis is associated with an increased production of a wide range of cytokines (Firestein et al, 1990; Dayer & Fenner, 1992; Kunkel et al, 1996; Feldmann et al, 1996 &1999; Steiner et al, 1999). Cytokines such as IL-1 and TNF-α produced by activated synoviocytes, mononuclear cells or by articular cartilage significantly up-regulate matrix metalloproteinase (MMP) gene expression. MMPs form a family of enzymes capable of degrading various extracellular matrix (ECM) components (Mauviel, 1993; Feldmann et al, 1996), as mentioned above in section 1.1.2.

High levels of IL-1, TNF-α, IL-6, IL-8, IL-15, IL-18, transforming growth factor-beta (TGF-β), granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein (MIP-1), and monocyte-chemoattractant protein (MCP-1) are detected in RA synovial tissue. TNF-α may be the major cytokine involved in the inflammatory process, whereas IL-1β is a key mediator with regard to cartilage and bone destruction (Firestein et al, 1990; Dayer & Fenner, 1992; Kunkel et al, 1996; Feldmann et al, 1996 &1999; Steiner et al, 1999). Analysis of the synovial cell infiltrate in early RA revealed a positive correlation between the disease activity, the number of macrophages, IL-6, and TNF-α expression in the synovium, suggesting that macrophage-derived cytokines play an important role in progression of the disease (Tak et al, 1997). Successful drug treatment of patients with RA resulted in a decrease in inflammatory cytokine levels such as TNF-α and IL-1β (Chang et al, 1992; Thomas & Carrol, 1993; Arend & Dayer, 1995; Seitz et al, 1995; Moreland et al, 1997; Dolhain et al, 1998).

Anti-inflammatory cytokines are also present in RA synovium, including IL-1 receptor antagonist (IL-1ra), soluble TNF receptor (sTNFR), IL-10, IL-11, and IL-13, and T cell-derived cytokines such as IL-17 and interferon beta (IFN-β) and low level of IL-4 (Firestein et al, 1990; Dayer & Fenner, 1992; Kunkel et al, 1996; Feldmann et al, 1996 & 1999; Steiner et al, 1999).

1.3.1 Proinflammatory cytokines: INTERLEUKIN-1 & TUMOUR NECROSIS FACTOR-α

Interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α) are thought to play a key role in the inflammation and joint damage that occur in RA. The pathologic effects caused by these cytokines include leukocytic infiltration leading to synovial hyperplasia, cell activation, cartilage breakdown, and inhibition of cartilage matrix synthesis. Moreover, IL-1 and TNF-α increase the expression of adhesion molecules (such as intracellular adhesion molecule-1 (ICAM-1), prostaglandin E_2 (PGE_2), MMPs (such as, collagenase, stromelysin and gelatinases) (Mauviel et al, 1993) chemokines (such as IL-8), and other pro-inflammatory cytokines (such as IL-6) (Dayer & Fenner, 1992; Arend & Dayer, 1995; Feldman et al, 1996; Mino et al, 1998). IL-1 shares many biological functions with TNF-α and is a potent factor in inducing damage to cartilage and bone, because it induces proteoglycan degradation and inhibits proteoglycan synthesis. Proteoglycans are important structural constituents of connective tissue, particularly of cartilage (van de Loo et al, 1995). Two distinct TNF-α receptor (TNF-αR) types have been demonstrated, 55-kDa (TNF-R1) and 75-kDa (TNF-R2) (Loetscher et al, 1991; Dayer and Fenner, 1992). Similarly, two types of IL-1 receptor (IL-1R) have been well studied in great detail (discussed in section 1.3.1.3).

In addition to the function of IL-1 in disease states, IL-1 has a wide range of normal functions. It is involved in proliferation and differentiation of T cells, B cells and fibroblasts and also stimulates vascular endothelial cells to secrete colony-stimulating factors. IL-1 may cause lymphocytes to release IL-12 and other cells to release IL-6 and also may induce IL-2 receptor on T cells. Moreover, IL-1 up regulates other inflammatory cytokines such as lymphocyte growth factors, chemokines, and mesenchymal growth factors (Arend & Dayer, 1995; van de Loo et al, 1995; Dinallero, 1996; Crofford et al, 1997; Arend, 2002 Vervoordeldonk & Tak, 2002).

Macrophages are the principal cells producing IL-1 and TNF-α. It is not clear yet which mechanism controls this production, however at least two major pathways exist: 1) induction of cytokine release by T and B lymphocytes, mast cells, or soluble factors (e.g. immune complex or other cytokines) and 2) induction by direct contact between the macrophages and activated T-lymphocytes (discussed later) (Firestein et al, 1990; Vey et al, 1992; Isler et al, 1993; Lacraz et al, 1994; Kunkel et al, 1996; Feldmann, 1996 & 1999; Steiner et al, 1999; Arend, 2002; Vervoordeldonk, and Tak, 2002). IL-1 and TNF-α are usually synthesised and secreted simultaneously, although their production is controlled by different mechanisms (Dayer & Fenner, 1992).

TNF-α is one of the proinflammatory cytokines that plays a pivotal role in RA. It is produced intracellularly (by macrophages, monocytes, lymphocytes and transformed cell lines of haemopoietic and non haemopiotic origin) as a 26-kDa propeptide and is also present in the cell membrane in an active form. The active form is a trimer with a subunit of 17-kDa. Like IL-1β, LPS induces the production of TNF-α and TNF-β. TNF-α/β can also be stimulated by other cytokines such as IFN–γ, IL-1, CSF and IL-

17 (Jovanonic et al, 1998). TNF-α has a wide range of functions; it induces IL-1β and other proinflammatory cytokines such as GM-CSF, IL-6 and IL-8 production. Moreover TNF-α up regulates expression of HLA-DR and IL-2R (Brennan et al, 1989; Feldman et al, 1996). There are two TNF-α inhibitors that have been found in urine of some febrile patients, 33kDa and 23 kDa named as TNF-α-INH (Seckinger et al, 1989; Abe et al, 2001).

Therapeutic approaches in RA have focused mainly on TNF-α; anti-TNF-α therapy shows efficacy in controlling signs and symptoms in most patients with RA. Clinical investigation in which the activity of TNF-α in RA patients was blocked by intravenously administrated Infliximab (monoclonal antibody against TNF-α) provided evidence that TNF-α regulates IL-1, IL-6, IL-8, MCP-1, vascular endothelial growth factor production, recruitment of immune and inflammatory cells into the joints, angiogenesis, and reduction of serum levels of MMP-1 and 3. Anti-TNF-α, when used in combination with methotrexate, resulted in significant improvement compared to methotrexate alone, (Brennan et al, 1989; Maini et al, 1998; Breedveld, 1999; Feldmann & Maini, 1999; Harriman et al, 1999; LaDuca et al, 2001).

1.3.1.1 IL-1 family

Three members of the IL-1 family have been studied intensively, including IL–1α, IL-1β and IL-1 receptor antagonist (IL-1ra) (reviewed by Dinarello, 1996). The genes for all three members of the IL-1 family, as well as for IL-1 receptors (IL-1R) type I and type II are located on the long arm of chromosome 2. The genes of IL-1α, IL-1β and IL-1ra show differential expression; for example, the IL-1β gene possesses a

TATA box, whereas IL-1α does not (Arend, 1993). IL-1β is regulated by at least two different signalling pathways, as demonstrated by differential induction with LPS or phorbol ester. Stimulation with LPS provokes a transient expression of IL-1β mRNA, while stimulation with phorbol ester gives rise to a more stable mRNA (Fenton et al, 1988). Moreover, complement component C5a also acts as an inducer of IL-1β transcription, however, the gene translation requires additional stimulus, such as LPS (Schindler et al, 1990). IL-1β gene transcription has been extensively investigated in response to LPS stimulation, as an immediate and potent inducer of IL-1β from monocytes (reviewed by Dinarello, 1996).

Both IL-1α and IL-1β are synthesized as intracellular proteins and released to act on target cells by binding to cell surface receptors (IL-1R). IL-1β requires proteolytic cleavage to produce the active form of the ligand; IL-1β is initially synthesised as a 31-kDa-precursor peptide, which is converted to the active form (17.5-kDa) by IL-1 converting enzyme (ICE, caspase-1) (Kostura et al, 1989; Cerretti et al, 1992). ICE acts to cleave the pro-IL-1β molecule specifically between the aspartic acid 116 and alanine 117 residues, thus releasing the 153 amino acid COOH terminal that constitutes the active cytokine (Black et al, 1989; Kostura et al, 1989).

ICE activity has been detected in monocytes and the human monocytic cell line THP-1 (Miller et al, 1993). Although stimulation of cells with lipopolysaccharide (LPS) or other stimuli is required for secretion of IL-1β, the enzyme activity has been identified in both stimulated and un-stimulated cells. LPS is one stimulus capable of inducing ICE activity in addition to increasing pro-IL-1β transcription. The mRNA for the enzyme has been detected in several non-monocytic cell types, including T

cells, B cells, and neutrophils. Because some of these cell types do not contain detectable IL-1β mRNA, it has been suggested that the enzyme may have other substrates in addition to pro-IL-1β (Black et al, 1989; Kostura et al, 1989; Thornbury et al, 1992; William et al, 1998). There is more recent evidence that demonstrates that ICE is required for the production of the mature form of the distant IL-1 family member, IL-18 (Ghayur et al, 1997).

In addition, stimuli such as LPS that initiate synthesis of pro-IL-1β from monocytes and macrophages are not necessarily sufficient to produce the active form, but in fact few of the pro-IL-1 are externalised as mature active molecules (Hogquist et al, 1991; Chin & Kostura, 1993). Efficient post-translational processing requires that LPS-activated cells encounter a secondary stimulus such as ATP (Perregaux et al, 1996, 1998a,b and 2000) cytolytic T cells (Hogquist et al, 1991), or bacterial toxins (Walev et al, 1996). All these secondary effectors promote major change to the ionic composition of the IL-1-producing cell and these changes appear to be essential for productions of active cytokine (Perregaux et al, 1996, 1998a,b and 2000). Moreover, ATP is shown to promote formation of mature caspase-1(ICE) subunits in addition to mature IL-1β and to elicit release of mature products (Laliberte et al, 1999).

1.3.1.2 Regulation of interleukin-1

The activity of mature IL-1β, once released, is regulated by several mechanisms, including IL-1ra, soluble IL-1 type II receptors that are cleaved from the surface of the cells (IL-1sRII), a decoy receptor (IL-1RII), which lacks an intracellular domain and is therefore, not capable of signal transduction, (discussed later), (Colotta et al,

1993) and by natural autoantibodies to IL-1, particularly to IL-1α (Forslind et al, 2001).

IL-1ra is the most important physiological regulator of synovial IL-1 activity. It has very high affinity for IL-1RI; thus it blocks the access of IL-1α and IL-1β to the receptor, (discussed in section 1.3.3). Cleaved fragments of the IL-1RII receptor (IL-1sRII), also inhibits the action of IL-1 by binding to circulating IL-1. Furthermore, IL-1 can be trapped on the cell surface by the membrane-bound decoy receptor, IL-1RII. Addition of IL-1ra and soluble IL-1sRII to human synoviocytes dramatically inhibited IL-1-induced MMP and PGE2 release (Colotta et al, 1994; Burger et al, 1995; reviewed by Burger and Dayer, 1995; Neumann et al, 2000). Moreover, TGF-β blocks IL-1 dependent proliferation of T cells and B cells (cited in Wright et al, 1991). IFN-β does not inhibit IL-1β directly but it stimulates IL-1ra production (Coclet-Ninin et al, 1997). Anti-TNF-α also has been shown to inhibit IL-1 production (Brennan et al, 1989).

1.3.1.3 Interleukin-1 receptors

The IL-1 receptor (IL-1R) family consists of a number of related receptor molecules; IL-1RI and IL-1RII are most important to be mentioned in this section. IL-1R1 is an 80-kDa protein, found mainly on T cells and fibroblasts. This receptor forms a complex with IL-1R accessory protein (IL-1R AcP) to bind IL-1β with high affinity. Antibodies to IL-1R AcP, which block binding to IL-1R1, also block IL-1β binding and signal transduction (Greenfeder et al, 1995). IL-1R acts as the primary effector receptor, after ligation with its ligand (IL-1) initiates an efficient amplification of

signal transduction and a rapid activation of NF-κB (Akahoshi et al, 1988, Ye et al, 1993).

IL-1RII is a 68-kDa protein, found in neutrophils, B cells, monocytes, and bone marrow cells (Chizzonite et al, 1989, reviewed by Dinarello, 1996). There is evidence to suggest that IL-1RII acts as decoy receptor, as there is no triggering of NF-κB induction when IL-1 binds to it (Sims et al, 1993; Colotta et al, 1994; Attur et al, 2000). It has been found that soluble IL-1RII significantly inhibited IL-1β-induced nitric oxide (NO) and / or PGE_2 production in chondrocytes, synovial and epithelial cells (Attur et al, 2000). Furthermore, IL-1ra was found to bind with high affinity to type I IL-1R, but not to the type II receptor (Stylianou et al, 1992), and also IL-1RII, but not IL-1RI is susceptible to proteolytic shedding, a finding consistent with the decoy function of this molecule (Orlando et al, 1999).

Other members of the IL-1R family, which do not bind to IL-1, include IL-1 receptor related protein (IL-1Rrp), and accessory protein-like receptor (AcPL), (Kumar et al, 1995; Born et al, 1998).

1.3.2 OTHER PROINFLAMMATORY CYTOKINES

IL-6 is a multifunctional cytokine that plays an important role in immune and inflammatory responses as it is elevated in inflammatory diseases. IL-6 activates T and B cells, and induces chemokine expression on endothelial cells. In addition to these inflammatory actions of IL-6, it also has anti-inflammatory functions, including, induction of anti-inflammatory cytokines such as IL-1ra and acute phase reactants, induction of protease inhibitors in RA fibroblast-like synoviocytes, inhibition of

cytokine production such as IL-1, TNF-α, IL-12, inhibition of adhesion molecules expression, and inhibition of protease expression. Levels of IL-6 are highly elevated in RA, especially in the synovial fluid and tissue during active disease and correlate with degree of joints damage, but its direct association in joint destruction still controversial. In RA, IL-6 is mostly produced by fibroblast-like synoviocytes and partly by other cells presents in RA synovium, such as macrophages. IL-6 works by regulating the expression of immune/inflammatory genes and regulating cell proliferation, differentiation and survival (reviewed by Tilg et al, 1997; Ahmed et al, 2000; Deon et al, 2001).

IL-12 has been found in the synovial membrane of patients with RA, suggesting that IL-12 may play an important immunoregulatory role in RA. It is a potent inducer of cell-mediated immunity by promoting differentiation of Th0 cells into Th1 type cells, and activating natural killer (NK) cells and $CD8^+$ cells. In RA, IL-12 acts synergistically with IL-18 in the generation of Th1 responses. IL-12 is produced by activated macrophages, dendritic cells, and to lesser extent by granulocytes. Interferon-γ production by RA synovial tissue cells was potently and selectively enhanced by IL-12 (Morita et al, 1998; Sakkas et al, 1998).

Interleukin-15 serves as a powerful T-cell chemoattractant and activator and induces B-cell maturation and isotype switching. It activates neutrophils and NK cell proliferation, cytotoxicity and cytokine production, and regulates NK cell/ macrophage interaction. Activation of T cells by IL-15 increases their ability to stimulate IL-1, TNF-α, IL-8 and MCP-1. IL-15 is produced by macrophages and maybe by other cells such as keratinocytes and synoviocytes, thus it may be involved

in self-perpetuating pro-inflammatory cytokine production through re-stimulation of T cells. IL-15 expression is significantly increased in RA synovium and in the synovial fluid compared with reactive arthritis and osteoarthritis (Thurkow et al, 1997; Fehniger & Caligiuri, 2001). Fibroblast-like synoviocytes isolated from joints of patients with RA secretes large amounts of IL-15 that may be increased after stimulation with TNF-α and IL-1β (Harada et al, 1999).

IL-17 is produced by T helper cells (Yao et al, 1995; Aaravak et al, 1999) and may play an important role in mediating inflammation in RA. It stimulates the production of IL-1β and TNF-α from macrophages and IL-6, IL-18, granulocyte colony stimulating factor (G-CSF) and PGE_2 from endothelial cells and fibroblast stromal cells, and NO from human osteoarthritic cartilage (Attur et al, 1997), thus contributing to the inflammatory response. Moreover, it induces neutrophil recruitment through chemokine release and stimulation of granulopoiesis. Furthermore, IL-17 increases expression of NF-κB, a transcription factor essential for proinflammatory cytokine secretion. Administration of IL-17R with sTNFR and sIL-1RII showed a significant decrease in synovial inflammation and bone resorption in rats, (Jovanovic et al, 1998; Shalom-Barak et al, 1998; Shin et al, 1998; Chabaud et al, 1999; Vervoordeldonk et al, 2002).

Interleukin-18 is a cytokine of the IL-1 family; like IL-1β the pro IL-18 lacks a signal peptide and requires ICE for cleavage and release of the active form (Ghayur et al, 1997). Although IL-18 and IL-1β are members of the same family, constitutive gene expression, synthesis, and processing are different for the two cytokines (Puren et al, 1999; Kashiwamura et al, 2002). IL-18 mRNA expression has been identified in

various cells, including macrophages, dendritic cells, Kupffer cells, kerotinocytes, chondrocytes, fibroblast-like synoviocytes, and osteoblasts. The IL-18 receptor consists of two subunits, α and β, which are members of the IL-1 receptor family (Torigoe et al, 1997; Born et al, 1998; Kashiwamura et al, 2002). IL-18R is expressed on T and B cells, NK cells, macrophages, fibroblast-like synoviocytes, neutrophils, and chondrocytes. IL-18 is a strong inducer of IFN-γ production by T cells and enhances the cytotoxicity in acquired and innate immunity. It also induces the production of the CC and CXC chemokines, TNF-α, IL-1β, GM-CSF, IL-2, IL-4, IL-5, IL-10, IL-13, PGE2 (Maclnnes et al, 2000; Jablonska et al, 2001; Kashiwamura et al, 2002; Vervoordeldonk et al, 2002) and adhesion molecules such as ICAM-1 (Yoshida et al, 2001). IL-18 works in synergy with IL-12 in the induction of Th1 cells because of the reciprocal regulation of the receptors (Barbulescu et al, 1998). Recently, IL-18 has also been associated with Th2 response, angiogenesis and neutrophil activation, suggesting a broader role for IL-18 (Mclnnes et al, 2000; Kashiwamura et al, 2002; Vervoordeldonk et al, 2002). Moreover administration of IL-18 promoted the development of collagen induced arthritis. In addition to that, IL-18 binding protein (IL-18BP) has been described as a novel modulator of the Th1 cytokine response. In an animal model, IL-18 blockade through polyclonal antibodies or recombinant human IL-1BP significantly reduced disease activity (Plater-Zyberk et al, 2001). Finally, neutralizing IL-18 monoclonal antibodies have the potential to become valuable tools for therapeutic approaches in vivo (Lochner et al, 2002).

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GM-CSF normally acts as a growth factor for monocytes as a part of haemopoiesis, but can also activate macrophages and monocytes and induce the expression of HLA-class II, thus it may contribute to the inflammatory process (Alvaro-Garcia et al,

1989). Macrophages Inflammatory protein (MIP-1α/β) are involved in the migration of T cells and immature dendritic cells, and may play an important role in RA and inflammation since it is upregulated by IL-1β and TNF-α and inhibited by IL-4 and IL-13 (Chabaud et al, 2001).

1.3.3 Anti-inflammatory cytokines: IL-1 RECEPTOR ANTAGONIST

The proinflammatory effects of IL-1 are counterbalanced by IL-1 receptor antagonist (IL-1ra). IL-1ra exists in 3 primary isoforms, a secreted form of 17-kDa (sIL-1ra) and both 18-kDa and 16-kDa intracellular forms, icIL-1raI and icIL-1raII respectively. It has been shown that introducing IL-1ra in animal models of arthritis causes reduction in disease severity (Burger & Dayer, 1995; Dayer & Fenner, 1996; Malyak et al, 1998). While sIL-1ra and icIL-1raI possess an equal IL-1R binding affinity, icIL-1raII is approximately 5 fold less effective at binding IL-1R (Arend et al, 1998).

The human IL-1ra gene is located on the long arm of chromosome 2, close to the region for IL-1α, IL-1β, IL-1RI, and IL-1RII. The gene structure for human IL-1ra is similar to those for IL-1α and IL-1β; however, IL-1ra complementary DNA (cDNA) contains four exons and three introns, whereas, cDNA for the active form of IL-1α and IL-1β contains three exons and two introns (Arend, 1993). The separate 5' regulatory region for both soluble and intracellular form of IL-1ra has been isolated, and some of the cis-acting DNA region involved in regulation of transcription has been characterized (Jenkins et al, 1997). The promoter region for sIL-1ra possesses one inhibitory LPS response element (LRE), and additional 3 positive response-binding sites (LRE1, LRE2, LRE3). LRE1 was further identified as a binding site for NF-κB and LRE2 a binding site for PU.1 (essential for LPS induced production of IL-

1ra or other cytokines) (Smith et al, 1994 & 1998; Arend et al, 1998). LRE3 has been shown to bind STAT6 and mediates IL-4-induced IL-1ra production (Ohmori et al, 1996).

The production of IL-1β and IL-1ra is differentially regulated; stimulation with LPS induced both IL-1ra and IL-1β, while IgG stimulation induced only IL-1ra production. IL-1ra is up regulated by variety of stimuli including, IL-1, IL-2, IL-3, IL-4, IL-6, IL-10, IL-13, TGF-β, IFN-α, IFN-γ, and direct contact with activated Th2 cells (discussed later) (Arend et al, 1991 & 1998; Chizzolini et al, 1997).

Raised levels of plasma IL-1ra have been detected in patients with juvenile arthritis (De Benedetti et al, 1995), RA (Chikanza et al, 1995), polymyositis (Gabay et al, 1994), and systemic lupus erythematosus (SLE) (Suzuki et al, 1995). High levels of IL-1ra have also been detected in synovial fluid of patients with RA. However, RA patients exhibit a lower IL-1ra to IL-1β ratio than patients suffering from osteoarthritis (Chikanza et al, 1995). This indicated that a high level of IL-1ra is essential to reduce the effect of IL-1. *In vitro* experiments have revealed that an excess of 100 times the amount of IL-1ra is required to inhibit IL-1β activity, whereas *in vivo*, 100-2000 times more IL-1ra is needed (Arend, 1993; Feldman et al, 1996). However, IL-1ra production by RA synovial cells is deficient relative to total IL-1 production (Firestein et al, 1994).

The capacity of IL-1ra to reduce *in vitro* and *in vivo* cartilage degradation, MMP production and the progression of inflammatory diseases such as RA has elicited much attention in therapeutic approaches, particularly in gene therapy (reviewed by

Krishnan, 1999; Abramson & Amin, 2002; Dayer & Bresihan, 2002). The use of recombinant human IL-1ra (rHIL-1ra) resulted in a significant reduction in joints erosion, and limited decrease in inflammation was also observed. rHIL-1ra was found to be safe and effective (Campion, 1996; Bresnihan et al, 1998 & 2001; Breedveld, 1999; Vervoordeldonk et al, 2002). Moreover, treatment of RA with IL-1ra resulted in reduced mononuclear infiltration of synovial membrane, which may represent the *in vivo* inhibition of biologically relevant IL-1-mediated pathogenic effects (Cunnane et al, 2001).

1.3.4 OTHER ANTI-INFLAMMATORY CYTOKINES

In addition to IL-1ra, there are several inhibitory cytokines that play a critical role in modulation of proinflammatory cytokines. Transforming growth factor-β (TGF-β) is a multi functional cytokine that exhibits both stimulatory and inhibitory effects upon a range of cell types. *In vitro*, TGF-β isoforms exert effects that can be grouped into three broad areas: modulation of inflammatory cell function, growth inhibition and differentiation, and control of extracellular matrix production. Elevated levels of TGF-β have been found in the synovial fluid of RA patients. The inhibitory effect of TGF-β results in inhibition of T and B cell proliferation and cytokine production (Ranges et al, 1987; Coffman et al, 1989). Interestingly injection of TGF-β into joints of normal rats induced rapid leukocyte infiltration into the synovium, while the administration of anti-TGF-β decreased the existing inflammation (Allen et al, 1990). Finally, TGF-β can inhibit the induction of MMP and partially inhibits IL-1 production, but is capable of inducing tissue inhibitor of metalloproteinase (TIMP) secretion (Wright et al, 1991; reviewed by Letterio & Roberts, 1998).

Interleukin-4 (IL-4) is a potent mediator able to shift the balance of Th1/Th2 cells, towards Th2 cells, and is produced specifically by Th2 cells. IL-4 antagonizes Th1 responses by direct inhibition of IFN-γ production by activated T cells. IL-4 decreases monocyte/macrophage cytotoxicity, cytokine production (including TNF-α, IL-1 and IL-6) and IL-15-induced cytokine production (reviewed by Burger & Dayer, 1995). In addition, IL-4 induces IL-1ra production (Ohmori et al, 1996) and upregulates the expression of the ''decoy receptor'' thus exerting an anti-inflammatory response (Collotta et al, 1993). IL-4 also suppresses biosynthesis of matrix metalloproteinases (MMPs) in alveolar macrophages. IL-4 can also inhibit the production of superoxide in macrophages (Abramson & Gallin, 1990, reviewed by Burger & Dayer, 1995).

Like IL-4, IL-10 displays immunosuppressive functions, since it down-regulates the production of IL-1, IL-6, IL-8, TNF-α, GM-CSF, and the expression of MHC class II on monocytes/macrophages. Furthermore, IL-10 inhibits the production of MMP by macrophages activated with LPS and stimulates the production of TIMP. B cells, monocytes/macrophages and T cells (T regulatory and Th2 cells) produce IL-10. Substantial amounts of IL-10 have been detected in RA synovium and synovial fluid but insufficient to reduce the effect of IL-1. Neutralization of endogenously produced IL-10 in RA synovial membrane cultures resulted in a two-to threefold increase in the protein levels of proinflammatory cytokines TNF-α and IL-1β, and addition of exogenous recombinant IL-10 to the RA synovial membrane cultures resulted in a two-to threefold decrease in the levels of TNF-α and IL-1β (Katsikis et al, 1994, Parry et al, 2000; Vervoordeldonk et al, 2002).

Interleukin-11 is a pleiotropic cytokine that regulates the growth and development of haematopoietic stem cells and decreases the proinflammatory mediators of cytokine and NO production. IL-11 has several biologic functions related to inflammatory events in RA; it is capable of inducing B cell differentiation (Anderson et al, 1992), production of acute phase reactants (Baumann et al, 1991), and osteoclast differentiation (Blair et al, 2004). It was suggested that IL-1 and TNF-α-induced osteoclast activation might be partly mediated by IL-11 (Romas et al, 1996). In addition, IL-11 has anti-inflammatory effects, especially on macrophages and fibroblasts and suppresses the expression of IL-12, IFN-γ, TNF-α , adhesion molecules, and proteases. IL-11 has been detected in the serum, synovial fluid, and synovial membrane of patients with RA (Okamoto et al, 1997). Treatment with recombinant human IL-11 (rH-IL-11) decreased the production of TNF-α and IL-1β and NF-κB- binding activity in RA synovium in vitro. Blockade of endogenous IL-11 resulted in a 2-fold increase in TNF-α levels and the addition of IL-11 inhibited TNF-α, MMP-1 and MMP-3 production. In addition, IL-11 stimulated MMP inhibitor levels in RA synovial tissue (Hermann et al, 1998).

IL-13 can also be considered as an inhibitory cytokine since it decreases the production of IL-1β, TNF-α, IL-8, MCP-1, MIP-1α, PGE_2, and epithelial neutrophil activating peptide-78 (ENA-78) (Woods et al, 2000). IL-13 has been detected at low concentration in rheumatoid synovium and synovial fluid. IL-13 has a homology with IL-4, and is considered as a Th2-like cytokine. IL-13 has profound effects on monocytes in vitro, deactivating them and acting in a complex manner to change monocyte morphology, phenotype, functions, and cytokine production (Woods et al, 2000; Vervoordeldonk et al, 2002). In addition to the anti-inflammatory functions of

IL-13 and IL-4 (mentioned above), both cytokines independently modify immune and inflammatory responses in several ways including enhancing monocyte/macrophage and endothelial cell expression of adhesion molecules, increasing macrophage expression of molecules associated with APC and T cell co-stimulation, and stimulating production of anti-inflammatory molecules such as the IL-1RII (Colotta et al, 1994) and IL-1ra (Muzio et al, 1994).

1.4 The role of T lymphocytes in RA

The role of T lymphocytes in the production of cytokines and development of RA has been debated at considerable length, with disagreements over their importance in the initiation and progression of inflammation. Based on animal studies it has been shown that the first cells to infiltrate the tissue are T lymphocytes, suggesting a pathogenic role for these cells. However, the mechanisms underlying the extravasations of T cells into the joints are still unclear. Some cytokines released by T cells such as IL-4, IL-10, IL-13, and TGF-β have predominantly anti-inflammatory effects, this leads to the notion that T cells exert their pathological effect through direct cellular contact with monocytes-macrophages. This interaction leads to IL-1β and TNF-α production, a process that is potentiated by many other factors including IFN-γ, IL-15, IL-17, and IL-18. In RA, IL-1β and TNF-α stimulate connective tissue cells such as fibroblasts and synoviocytes to produces MMPs, which degrade the extracellular matrix component (e.g. collagens and proteoglycans). Also in RA, T lymphocytes located in the peripheral blood primarily express the CD45RA (naïve pool) isoform, whereas, most synovial T lymphocytes express the CD45RO (memory pool) isoform. Also, increased expression of HLA-DR molecules has been associated with synovial T cells, although this observation remains controversial (Panayi et al, 1992; Lacraz et

al, 1994; Summers et al, 1994; Isler et al, 1995; Chizzolini et al, 1997; Miossec and Berg, 1997; Vey et al, 1997; Burger & Dayer, 2002; Brennan & Foey, 2002; Miossec, 2003).

Th1 cells release IFN-γ, and to a lesser extent IL-2 and IL-12, promoting a proinflammatory response. Moreover, IL-17 (Aarvak et al, 1999) and IL-18 (Barbulescu et al, 1998) have been associated with Th1 responses. On the other hand, Th2 cells secrete IL-4, and sometimes IL-5, IL-6, IL-10 and IL-13, and enhance anti-inflammatory responses (Constant & Bottomley, 1997).

Moreover, several subsets of regulatory T cells, the Tr cells, have been identified that appear to be distinct in their function and phenotype from the Th1 and Th2 cells. Type 1(CD4+/CD8+) Tr cells (Tr1) secrete high levels of IL-10 and IL-5 and low-to-moderate level of TGF-β, and little or no IL-2, IL-4, or IFN-γ. Tr1 proliferate poorly following TCR stimulation, have immunosuppressive properties, and have been shown to prevent the development of Th1-mediated autoimmune disease. Moreover, functional studies indicate Tr1 cells and other Tr populations may help terminate Th1-related inflammatory response to pathogens, tumours, and alloantigens. Type 3 (Th3) ($CD4^+$) cells primarily secrete TGF-β, and since this cytokine acts on multiple cell types, Th3 cells may be involved in many roles of immune regulation. It has been shown that Th3 cells inhibit immune response through cellular contact. Another type of regulatory T cells are called $CD4^+$ $CD25^+$ Tr cells. These cells also exhibit immunosuppressive effects, and inhibit IL-2 production via cell-cell contact and expression of inhibitory co-stimulatory molecule CTLA-4 (Groux & Powrie, 1999;

Groux, 2001; McGuirk and Mills, 2002; Kidd, 2003; Foussat et al, 2003; Cottrez & Groux, 2004).

The differentiation of Th1 cells occurs in response to a combination of IL-12 and IFN-γ. The role of IFN-γ is the inhibition of Th2 outgrowth, while IL-12 directly augments Th1 differentiation, having no inhibitory effects upon Th2 cells (Seder & Paul, 1994). Th2 cell differentiation is primarily under the control of IL-4 (Swain et al, 1994). Both IL-6 (Rincon et al, 1997) and PGE_2 (reviewed by Harris et al, 2002) have also been implicated in the Th2 response, IL-6 by stimulating IL-4 production from naïve T cells, and PGE_2 by inhibiting both the release of IL-12 from dendritic cells and the release of IFN-γ from T cells (Hilkens et al, 1997, reviewed by Harris et al, 2002).

T cells may play a critical role in RA progression. In the peripheral circulation of RA patients, increased numbers of activated T cells have been observed. Activated T cells are also present in abundance in the rheumatoid synovium. These cells express various cell surface molecules such as Very Late Activation Antigen (VLA-1) and Leukocyte Function Antigen-1 (LFA-1) at high densities. In addition to that, cells express the early activation antigen CD69 (Panayi et al, 1992 and 1999a; Weyand, 2000). Additional evidence implicating T cells in the pathogenesis of RA comes from animal studies; in experimental models of rheumatoid-like inflammatory arthritis such as collagen-induced arthritis, synovitis can be generated in susceptible hosts via the transfer of T cells from affected animals (Holoshitz et al, 1984). Perhaps the most convincing evidence of the role of T cells in RA has resulted from patient studies with various types of therapeutic interventions. These therapies, which involves

elimination or interference with T cells resulted in regression of the rheumatoid inflammation, for example administration of anti-CD4 monoclonal antibodies resulted in dose-dependent reduction in disease activity that correlate with $CD4^+$ T cells coating with the antibodies (Wagner et al, 1994; Moreland et al, 1997; Klimiuk et al, 1999; Taylor, 2003). Among the subsets of T cells, $CD4^+$ T cells have been suggested to play a critical role in RA. As mentioned above, these cells are present in abundance in the rheumatoid synovium and represent the predominant T cell subset in the organised perivascular cellular aggregate (Harris et al, 1990; Steiner et al, 1999). Further evidence implicating $CD4^+$ T cells comes from genetic studies. Because $CD4^+$ T cells recognise antigen bound to MHC class II antigens, the association of RA with expression of specific class II MHC molecules strongly implies a role for $CD4^+$ T cells in RA (Gregersen et al, 1987&1996; Thomas & Quinn, 1996).

There is a recent demonstration implicating Th1/Th2 balance in the pathogenesis of RA. Several studies have demonstrated a shift towards Th1 cells as determined by cytokine profiles and chemokine receptors present on T cells isolated from rheumatoid synovial joints, often accompanied by an increase in the proportion (relative to Th1) of Th2 cells in the peripheral blood due to preferential tissue infiltration by Th1 cells (Dolhain et al, 1996; Miossec & Berg, 1997). As mentioned above, Th1 cytokines (IL-2, IFN-γ) favour T cells mediated cellular immunity and cytotoxicity, and activate monocytes, which leads to proinflammatory cytokine production such as IL-1β. Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) favour B cells mediated-humoral immunity and deactivate monocytes consequently lead to an anti-inflammatory response (Dolhain et al, 1996; Chizzolini et al, 1997; Miossec & Berg,

1997; Vey et al, 1997). These findings might have implication for development of new therapies for RA.

In addition to the production of a range of cytokines, Th1 and Th2 cells differentially express chemokine receptors. Th1 cells express CCR2, CCR5 and CXCR3, with CCR1 and CXCR5 expressed on a specific subset of cells. Th2 cells express CCR2, CCR3 and CCR4 with a subset of cells expressing CXCR5 (reviewed by Sallusto et al, 1998). Chemokine receptors play a critical role in migration of lymphocytes into inflamed sites. In RA patients, much attention has been focused on the expression of CCR5, CXCR3 (Th1 markers) and CXCR4 (Th2 marker) (Syrbe et al, 1999). Recently it has been reported that expression of CCR5 and CXCR3 is up regulated on the synovial T cells (Gomez-Reino et al, 1999; cited in Nanki et al, 2000).

1.4.1 T-lymphocyte activation

1.4.1.1 TCR/CD3, CD28 & CD2

Complete activation of T cells requires ligation of the T cell receptor (TCR) by antigen-MHC complexes together with a co-stimulatory signal, delivered by APC. B7-1 (CD80) and B7-2 (CD86) provide important co-stimulatory signals through interaction with CD28 on T cells, but other interactions including those between adhesion molecules and their ligands, such as intracellular adhesion molecule-1 (ICAM-1)/LFA-1 or LFA-3/CD2, are also involved (June et al, 1994; Guinan et al, 1994).

Upon engagement of the TCR by antigen presented on MHC, the Src family kinase Lck is activated and then phosphorylates immuno-receptor tyrosine-based activation motifs (ITAMs) on the ε,δ,γ and ζ subunits of TCR. Phosphorylated ITAMs promote the recruitment and subsequent activation of another tyrosine kinase ZAP-70. ZAP-70 triggers phosphorylation of LAT and SLP-70, phosphorylation of tyrosine residues on LAT and SLP-70 results in recruitment of a number of other proteins involved in activation of the Ras pathway, calcium mobilization and cytoskeletal reorganization.

One important protein that is recruited to LAT upon TCR stimulation is phospholipase Cγ1 (PLCγ1). Activated PLCγ1 is essential for production of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate [Ins (1,4,5) P3] (Perlmutter et al, 1993). DAG activates protein kinase C (PKC), PKC activates MAPK such as p38, JNK, and ERK1/2. Phosphorylation of MAPKs leads to activation of transcription factors such as NF-κB (via IκB). Activation of NF-κB is dependent on the stimulation of the TCR and co-stimulation via CD28. The serine/theonine kinase and MAP kinase kinase kinase (MAPKKKs) participate in the activation of a regulator of NF-κB (IκB). IκB binds to NF-κB, phosphorylation of IκB causes release of NF-κB to be moved into the nucleus and activate transcription (Weiss et al, 1984; Cope et al, 2002; Kane et al, 2002). Ins (1,4,5) P3 binds to its receptor on endoplasmic reticulum (ER), causing release of calcium to the cytoplasm from calcium stores in the ER. This event triggers the opening of calcium- release-activated calcium (CRAC) channels at the plasma membrane, allowing influx of extracellular calcium (Sorrentino et al, 2001). Reduced intracellular Ca^{2+} signalling after TCR stimulation in peripheral blood and synovial T cells of RA has been reported. This reduction was correlated with decreased IL-2 production and T cell proliferation, suggesting that the changes in

magnitude of the intracellular Ca^{2+} signal may contribute directly to the functional aberration in RA T cells (Allen et al, 1995; Carruthers et al, 1996/2000), however, this observation still not well studied.

CD28, a 44-kDa member of the immunoglobulin superfamily, is constitutively expressed on the majority of $CD4^+$ T cells as a disulphide-linked homodimer. Its natural ligand is B7. Various studies have shown positive effects of CD28 co-stimulation (with TCR signal) on proliferation, cytokine production (e.g IL-2) and survival of T cells. This stimulation also induces secretion of other cytokines such as IFN-γ, IL-4, IL-8, IL-3, GM-CSF and IL2R (Guinan et al, 1994). Unlike the TCR signal, which depends on PKC, CD28 co-stimulation apparently does not require PKC; this indicates that stimulation via CD28 differs from that via TCR. However, like TCR signalling, CD28 activation triggers PI3 kinase activation (reviewed by Acuto & Michel, 2003). Association between CD28 and the NF-κB pathway has been suggested, by identification in the proximal IL-2 promoter of a response element similar to that recognised by NFκB. CD28-responsive elements, (CD28RE), confer responsiveness to anti-CD28 antibodies. Mutation or deletion of CD28RE abolished the increase in transcription mediated by CD28 (Kane et al; 2002). Finally, surface expression of β chemokine receptors CCR1, CCR2 and CCR5 are down regulated following CD28 stimulation (Carroll et al; 1997; Acuto & Michel, 2003).

Despite their activated phenotype, synovial T cells in RA patients show features of hyporesponsiveness after TCR/CD3 and CD28 stimulation. Hyporesponsiveness of synovial T cells has been associated with decreased levels of intracellular GSH, and treatment of those cells with NAC improved CD3 stimulation, while CD28

stimulation was unaffected (Maurice et al, 1998). This indicates that CD3 stimulation is redox sensitive. In addition, it has been shown that the synovial T cells of RA patients show severely impaired phosphorylation of the adaptor protein linker for activation of T cells (LAT), TCR-ζ, and $p56^{lck}$, crucial components of the TCR-mediated signalling pathway (reviewed by Salojin et al, 1998; Gringhuis et al, 2000; Cemerski et al, 2003), and also reduced protein tyrosine phosphorylation and Fyn expression (Ritter et al, 1997). There are several possible reasons for this phenomenon; the anatomy and cellular environment of the inflamed synovium are different from the lymph nodes, the hypoxic nature and the pH of the inflamed synovium has an effect on the T cells. Subcellular localization of LAT is sensitive to changes in the cellular levels of the antioxidant glutathione (Gringhuis et al, 2000). This suggests that the hypo-responsiveness of RA T cells may be due to their constant subjection to high levels of oxidative stress. Moreover, the cytokine milieu in RA synovial tissue is also different from that expressed in the lymph nodes due to primary interaction between macrophages and Th cells (reviewed by Cope, 2002).

Stimulation of T cells via CD2, using lectins (Phytohaemagglutinin, PHA), also causes a rise in cytoplasmic calcium levels, which is derived from both extracellular sources and from stores in the endoplasmic reticulum. CD2 stimulation follows the same pathway as CD3 stimulation that leads to the generation of IP3, which binds to intracellular membranes, causing mobilization of intracellular stores of calcium (Carruthers et al, 1996). Furthermore, CD2, CD3, and CD28 stimulation of RA patients T cells with tri-specific antibody induced higher level of IL-2 than normal T cells, and favours Th1 cytokines (such as IFN-γ). This tri-specific antibody (Tsab) was significantly more potent than PHA in stimulating IL-2 production by RA T cells.

However, in normal controls IL-2 secretion was not significantly greater with Tsab than PHA stimulation, indicating that stimulation via CD2 is very effective in cytokine induction (Wong et al, 2000).

1.4.1.2 BACTERIAL LIPOPOLYSACCHARIDE (LPS)

LPS is the main constituent of the outer leaflet of gram-negative bacteria, it consists of a saccharide part and lipid part, termed lipid A. The hydrophobic portion of lipid A has been identified as the endotoxic part of LPS. Low dose of LPS may be beneficial for the immune system, by causing immuno-stimulation and enhancing resistance to infection and malignancy. LPS has been used extensively for monocyte activation, and will be discussed in section 1.5.1.1 in greater detail (Ulevitch et al, 1995). LPS is also a potent stimulator of human T cell proliferation and Th1 associated cytokine production. It was shown that T-lymphocyte activation by LPS is strongly dependent on direct cell-cell contact of responding T lymphocytes with viable accessory monocytes, mainly represented by interaction of B7 with CD28 (Mattern et al, 1998 &1999). It was found that LPS or lipid A was able to induce $CD4^+$ and $CD8^+$ lymphocyte activation and DNA synthesis. Further analysis of LPS-responding T lymphocytes found them to express CD25 on their surface, and they are in the memory pool (CD4+/CD45RO+) as well as naïve pool (CD4+/CD45RO-). The response of LPS-stimulated T lymphocytes resembled an antigen-driven response with a low frequency of responding cells, and addition of monocytes/macrophages or other accessory cells such B cells or dendritic cells enhanced the response. This enhancement of T cell proliferation was found to be due to cell-cell contact between monocytes and T cells (Mattern et al, 1998). Moreover, blocking of the biological activity of IL-12 by a neutralizing anti-IL-12 mAb resulted in a clear reduction of T

cell proliferation, indicating that in LPS-induced T cell proliferation not only an interaction by direct cell contact was necessary, but also the release of soluble factors such as IL-12. IL-12 alone is not able to induce proliferation in resting T cells.

One function of monocytes during activation of T cells by LPS is to present a co-stimulatory signal. These signals have to be presented by living monocytes since paraformaldehyde-fixed monocytes failing to internalise and re-express antigen did not support LPS induced T lymphocyte proliferation. In addition to that, the interaction of LPS primed monocytes with T cells was not MHC-restricted, since primed autologous monocytes could be replaced effectively by primed heterologous monocytes and also antibodies to MHC-II had no effect in LPS-induced T cells proliferation. Furthermore, co-stimulatory signals through CD28 are required for optimal stimulation of human T lymphocytes by LPS, (Mattern et al, 1998, Brennan & Foey, 2002; Burger & Dayer, 2002; reviewed by Acuto & Michel, 2003). However, the significance of the activation of T cells by LPS, and the contribution of haematopoietic stem cells to this process remain to be investigated.

1.4.1.3 PHORBOL ESTER/CALCIUM FLUX

Lymphocytes can be activated by the synergistic action of protein kinase C binding phorbol ester (e.g. phorbol-12, 13-dibutyrate, (PDBu) and calcium ionophore (e.g. ionomycin), thus mimicking the action of DAG and IP3 respectively (Trunech et al, 1985; Berry et al, 1989). Phorbol esters mimic DAG activation of PKCs by binding to the same sites within the regulatory domain and inducing similar conformational changes. The DAG / phorbol ester binding sites have been mapped to two pairs of

zinc fingers in the regulatory domain (C1A and C1B). Structural studies demonstrated that in the presence of phorbol ester, this domain forms a hairpinlike hydrophobic structure that mediates PKC interactions with membrane lipids, thus phorbol ester binding promotes conformational changes that trigger catalytic activity. Production of second-messenger DAG in response of physiological stimuli is transient due to the rapid metabolic conversion of DAG. In contrast, phorbol esters are metabolically stable in most cells. As a consequence, PKC activation by phorbol esters is much more prolonged than the transient activation that occurs with physiological stimulation (Gopalakrishna et al, 2000).

Calcium ionophores such as ionomycin (Liu &Hermann, 1978) mimic Ins (1,4,5) P3. Ins (1,4,5) P3 binds to its receptor on endoplasmic reticulum (ER), causing release of calcium to the cytoplasm from calcium stores in the ER. This event triggers the opening of calcium-release-activated calcium (CRAC) channels at the plasma membrane, allowing influx of extracellular calcium (reviewed Sorrentino & Rizzuto, 2001).

Phorbol ester/Calcium ionophore can lead to increased responsiveness to IL-2 via upregulation of high affinity IL-2R, and induction of IL-2 release, in combination with a significant proliferative response (Stoeck et al, 1987). In addition, phorbol ester/calcium ionophore causes activation of NFκB, a transcription factors (Baeuerle & Henkel, 1994).

1.4.1.4 CD69, SURFACE RECEPTOR

CD69 has been found expressed on all peripheral blood monocytes, and is also one of the earliest cell surface molecules expressed after activation of T and B cells and other cells of haematopoietic origin (reviewed by Testi et al, 1994). It is thought to play an important role in production of IL-1β (Manie et al, 1993; Isler et al, 1993), and IL-15 (Ortiz et al, 1999) in RA. CD69 can act as a potent stimulatory molecule on the surface of human peripheral blood monocytes (De Maria et al, 1994). Moreover, anti–CD69 induced TNF-α production by human monocytes (Ramirez et al, 1996).

Activation via TCR/CD3, phorbol ester such as phorbol myristate acetate (PMA) or PDBu or via IL-2 receptor, results in activation of the Ras protein, which subsequently initiates CD69 expression and cell activation. These pathways may involve protein-tyrosine kinases (PTKs) and/or PKC. Cross-linking of CD69 on activated T cells was shown to generate an extracellular calcium influx (Maria et al, 1994), and upon simultaneous PKC stimulation, to induce the expression of IL-2, IFN-γ and TNF-α. Signal generation from CD69 in T cells requires the expression of the TCR/CD3, suggesting that CD69 may act in close sequence to TCR/CD3 signalling (reviewed by Testi et al, 1994).

1.5 The role of monocyte/macrophages in RA

The role of monocytes and macrophages in RA has been well established (Burmester et al, 1997; Kinne et al, 2000). Macrophages constitute a major part of both the normal and rheumatoid synovial lining, and are frequently filled with phagocytosed material (Zvaifler et al, 1995). Monocyte/macrophages have several roles that may contribute to the progression of RA. Synovial tissue macrophages and peripheral blood monocytes show signs of activation, reflected by the production of proinflammatory cytokines, such as TNF-α, IL-1, and MMPs. These factors play a crucial role in mediating joints destruction in RA. Moreover, it has been hypothesised that monocytes may introduce antigens into lymph nodes or joint tissue, thus it is possible that an antigen-incited process is not necessarily initiated in the synovial tissue, but rather may be imported.

Circulating autoantibodies, such as RF, anti-perinuclear antibodies and anti-keratin antibodies have been detected in RA (Corrigall & Panayi, 2002). Although the pathological role of these autoantibodies is not fully characterised, they may contribute to disease by forming immune complexes. Binding of IgG containing immune complexes to FcγRs expressed on monocytes and macrophages, leads to activation of these cells. This initiates a diversity of effector functions, such as phagocytosis, antigen presentations, antibody-dependent cellular cytotoxicity and the release of proinflammatory and tissue destructive mediators (Blom et al, 2000; de Stahl et al, 2002; Wijngaarden et al, 2003), as well as anti-inflammatory cytokines such as IL-1ra (Arend et al, 1991).

Macrophages in the cartilage-pannus junction also produce modest amounts of proteolytic enzymes, such as collagenase and stromelysin, gelatinase B and elastase. However, the destruction of cartilage and bone in RA is the result of complex cellular interactions, in which the macrophages play a crucial role in the pathogenic cascade, but are not necessarily the primary effector cells of matrix destruction (Mauviel, 1993). There are multiple ways by which macrophages interact with other cells to trigger the formation of invasive inflammatory pannus: macrophages are the main source of platelet-derived growth factor (PDGF) in RA synovium. This cytokine causes synovial fibroblasts to grow under anchorage-independent conditions, similar to transformed cells (Lafyatis et al, 1989). In addition, inflammatory cytokines (TNF-α, IL-1β) contribute to the generation of proteolytic enzymes by synovial fibroblasts. Also it has been shown that numbers of synovial macrophages correlate with articular destruction in RA (Mulherin et al, 1996; reviewed by Burmester et al, 1997).

Cytokines are crucial for directing the differentiation of the monocyte/macrophage system, (as mentioned in section 1.3). For example, TNF-α inhibits the differentiation of monocytes into macrophages and, together with GM-CSF, directs the precursor cells to differentiate into dendritic cells and to osteoclast cells, which greatly contribute to bone destruction in RA (Reid et al, 1992). Macrophages in RA synovial tissue are characterized by increased expression and transcription of IL-1β and TNF-α reflecting the activated state. However, not only the synovial macrophages but also peripheral blood mononuclear cells of RA patients showed elevated levels of these cytokines (Firestein et al, 1990; Dayer& Fenner, 1992; Kunkel et al, 1996; Feldmann et al, 1996 &1999; Steiner et al, 1999).

It is not known which factor(s) prime monocytes to subsequently locate in the specific organ systems. Two mechanisms may be involved: monocytes may be educated in the bone marrow to enter designated sites, or second, monocytes may bind to adhesion molecules present either physiologically or upon inflammation, and subsequently enter the respective tissues. Once monocytes have entered the tissue, they do not recirculate, but may undergo differentiation and activation events, for example, the formation of granulomas consisting of epithelioid and giant cells in synovial tissue or nodules of RA patients (Zvaifler, 1995; Burmester et al, 1997).

1.5.1 Mechanism of monocyte/macrophage activation

1.5.1.1 BACTERIAL LPS

LPS is a potent stimulant for monocytes and macrophages. It activates a cascade of inflammatory responses leading to increase secretion of cytokines such as TNF-α (Yao et al, 1997), IL-1 (Buras et al, 1998; Schumann et al, 1998), IL-6 (Tilg et al, 1997) and IL-8 (Tanaka et al, 1997). Also it has been demonstrated that LPS activates caspase-1 in the monocyte cell line (THP-1), freshly isolated peripheral blood monocytes, and in human umbilical vein endothelial cells (HUVECs) in a time-and dose-dependent manner. Caspase-1 activation is associated with cleavage of the IL-1β precursor protein that is followed by release of the mature IL-1β precursor protein (section 1.3.1) (Schumann et al, 1998). LPS has been shown to induce acute phase proteins (Haziot et al, 1988), a set of functionally diverse proteins thought to increase host defences.

LPS binds to a serum protein called LPS-binding protein (LBP) (Tobias et al, 1993; Schumann et al, 1996), LBP rapidly catalyses the transfer of LPS to membrane-bound

CD14 (mCD14) or soluble CD14 (sCD14). Although CD14 has been identified as an LPS receptor, it is not a transmembrane receptor and lacks intracellular domain (Wright et al, 1990; Ulevitch & Tobias, 1994; Pugin et al, 1998). The mechanisms by which CD14 mediates a transmembrane signal have remained elusive. It has been hypothesised that additional transmembrane receptors must act in concert with LPS-CD14 complexes to initiate the signalling process leading to LPS-induced cellular activation. Toll-like receptor-4 (TLR-4) has been associated with LPS responsiveness. TLR-4 requires additional molecules, MD2, which forms a complex with the extracellular domain of TLR-4 for effective LPS recognition (Lein et al, 2000; Akashi et al, 2001; Correia et al, 2002; Zarember et al, 2002). Moreover, it has been documented that LPS must initially bind to CD14 and is then transferred to the membrane where it associates with a complex of receptors, including TRL-4-MD2 (Correia et al, 2002, Triantafilou M & Triantafilou K, 2002). A recent discovery indicated that LPS-binding site involves heat shock proteins (Hsp) 70 and 90, CXCR4, and growth differentiation factor 5 (GDF5), all four molecules form a complex after LPS ligation. Moreover, Pfeilffer et al (2001) suggested a new LPS activation cluster composed of CD14, TLR, CD55, CD16a, CD11b/CD18, Fcγ-receptors, CD32 and CD64, Fcγ-RIIIa, CD36 and CD81 (Pfeilffer et al, 2001; reviewed by Triantafilou M & Triantafilou K, 2002). In conclusion, molecules involves in LPS binding site are complicated and depends on the cell type, for example, LPS binding site on B cells has been shown to engage TRL-4, RP105 and MD1, whereas in mast cells the binding site is found to be TLR-4 and MD2 (reviewed by Triantafilou M & Triantafilou K, 2002).

LBP is a 60kDa glycoprotein, which binds to the lipid A portion of LPS, and is a member of a family of lipid carrier proteins capable of transporting amphipathic molecules in aqueous environments. A major function of LBP is to enable LPS binding to either membrane or soluble CD14. LPS-LBP complex enhanced TNF-α and NO production. LBP also enhances LPS-induced upregulation of adhesion molecules in polymorphonuclear cells. Depletion of this protein from plasma markedly reduces LPS-induced cell activation (Wright et al, 1990; Ulevitch & Tobias, 1994).

The mechanism of internalisation of mCD14-bound LPS has not been well described. It may enter cells via clathrin-coated pits, or via the glycosylphosphatidylinisitol-anchored proteins. LPS then moves into intracellular compartments where the lipid A portion exerts its biological activity (Pugin et al, 1998). LPS has been shown to move from the plasma membrane to intracellular vesicles. There is also evidence that LPS may remain on the plasma membrane for long periods of time before its active form is released. The binding between CD14 and LPS, and subsequent internalisation of LPS has been linked to both the activity of LBP and the size of LPS aggregates involved with larger aggregate and presence of LBP resulting in an increase rate of internalisation (Kitchens & Munford, 1998; Kitchens et al, 1998).

Several signal transduction pathways have been associated with LPS-CD14-TLRs interaction. These signals are generally alike in that they result in the activation of transcription factor NF-κB, which leads to proinflammatory cytokine production. It has been shown that LPS treatment of monocytes (Weinstein et al, 1992 & 1993) and HUVECs (Schumann et al, 1996) leads to increased protein tyrosine phosphorylation

and MAP kinase activation, including ERK1/2, JNK and p38 (Weinstein et al, 1992 & 1993).

Alternative pathways involve phosphatidylinositol-3 kinase (PI3K), activation of phospholipase D (PLD), and PKC (Herrera-Veilt & Reiner, 1996). Activation of protein tyrosine kinases (PTKs) by LPS results in activation of PI3K, followed by stimulation of PLD by both PTKs and PI3K. DAG activates PKC, consequently leading to MAPK kinase activation (Buscher et al, 1995). In addition, activation with LPS leads to conformational changes in the TLR-4 cytoplasmic domain and subsequent recruitment of an adaptor named MyD88. MyD88 recruits downstream IL-1 receptor-associated kinases (IRAK). IRAK is then phosphorylated and dissociates from the receptor complex and recruits TNF-receptor-associated factor 6 (TRAF6) that in turn activates downstream kinases such as MAP kinases, ERK kinase kinase1 (MEKK1) and NF-κB inducing kinases (NIK) (Zhang & Ghosh, 2001).

Endogenous inhibitors of LPS signalling have been discovered, phosphatidylinositides are capable of binding to mCD14 (utilising the action of LBP), and inhibit monocyte activation by preventing binding of LPS (Wang et al, 1998). Tissue factor pathway inhibitor (TFPI) is a plasma protease inhibitor that plays an important role in feedback inhibition of the coagulation cascade. It is also able to bind to LPS and prevent interaction with both LBP and CD14 (Park et al, 1997). Blockade of NF-κB activity by excess IκB is able to completely inhibit proinflammatory cytokine and partially anti-inflammatory cytokine expression in response to LPS (Bondeson et al, 1999). This suggests that blockade of NF-κB, or preventing its activation, might be a useful tool in treatment of inflammatory disease.

1.5.1.2 1,25 DIHYDROXYVITAMIN D_3

The steroid hormone 1,25 dihydroxyvitamin D_3 (1,25 (OH) $_2$ D_3) is the active form of vitamin D3 produced exclusively in the kidney. It is an important regulator of bone and mineral metabolism, and has multiple effects on cells of the immune system. It plays a role in controlling the growth and differentiation of several cell lines such as the promonocyte lines THP-1 and U937 and lymphocytes (Lemire, 1992; reviewed by Ross et al, 1994). Of particularly importance to the field of proinflammatory cytokine studies was the discovery that 1,25 (OH) $_2$ D_3 was capable of stimulating myeloid cells to differentiate along a monocyte/macrophage lineage. This technique is now widely used in the study of proinflammatory cytokine production by promonocytic cell lines such as THP-1. 1,25 (OH) $_2D_3$ induces CD11b and CD14 expression on these cells and subsequently increase cytokine release such as IL-1β, TNF-α, and IL-6 in response to additional stimulation (Vey et al, 1992; Chizzolini et al, 1997; Landman et al, 1998). The mechanism controlling this differentiation is believed to be primarily due to an effect on gene expression (Obeid et al, 1990), and increased expression of calcium binding proteins (Roth et al, 1993).

1,25 (OH) $_2D_3$ exerts its activity on responding cells via vitamin D receptor (VDR). This receptor is expressed on a wide range of cell types including monocytes and activated lymphocytes (reviewed by Ross et al, 1994). A number of other immunomodulatory and immunosuppressant roles have been determined for 1,25 (OH) $_2$ D_3. It inhibits the transcription and secretion of IL-2 and the transcription of IFN-γ in T helper cells (Rigby et al, 1984), and also inhibits the generation of NK and cytotoxic T cells (Merino et al, 1989; Lemire, 1992). There is also a suppressive

effect on immunoglobulin production by B-lymphocytes; this may be due to the inhibitory effects on the Th-cell population (Iho et al, 1986; Lemire, 1992).

1.5.1.3 OTHER INDUCERS OF MONOCYTE ACTIVATION

Monocytes/macrophages can be activated via Fc receptors (FcR), FcγR (IgG) is directly relevant to monocyte/macrophage activation (Sutton & Gould, 1993). FcγR mediate a range of activities on monocytes/macrophages, including phagocytosis, endocytosis, and release of inflammatory cytokines and NF-κB activation (van de Winkle & Anderson, 1991; Tsitsikov et al, 1995). Cross-linking of FcγRIIIa is also able to stimulate TNF-α secretion in monocytes (Abrahams et al, 1998). Interestingly, elevated expression levels of FcγRIIa and IIIa has been reported in RA (Wijngaarden et al, 2003) and related to the disease severity and cartilage destruction (Blom et al, 2000; Brun et al, 2002).

Phorbol esters such as PMA trigger signal transduction cascades, due to their ability to mimic DAG, which activates PKC (Sharkey&Blumberg, 1985). Activation by phorbol esters induces expression of ICAM-1 (Dean et al, 1994; Kurosoka et al, 1998). Phorbol esters have also been used to induce differentiation of myeloid progenitor cells (such as THP-1) into more mature cells (Pedron et al, 1995) and induce proinflammatory cytokines such as IL-1β. Calcium flux, induced for example by calcium ionophores such as ionomycin or A3187, also activates monocytes, and stimulates IL-8 expression similar to LPS (Wilson et al, 1993). Incubation of peripheral blood myeloid-lineage cells with ionomycin or A23187 down regulated CD14 expression (Czerniecki et al, 1997).

1.6 The principle of T-lymphocyte-monocyte co-culture

T cells and monocytes are major sources of inflammatory mediators and can communicate with each other as well as with other cells. This communication is mediated by soluble factors and by direct cell-cell contact. Interactions between T cells and monocyte-macrophages result in the production of cytokines such as IL-1 and TNF-α. In RA, these cytokines stimulates connective tissues cells such as fibroblast-like synoviocytes to produces MMPs, which degrade the extracellular matrix components of the cartilage (e.g. collagens and proteoglycans) (reviewed by Burger & Dayer, 2002).

Direct contact with stimulated T lymphocytes is a potent mechanism which induces the production of pro-inflammatory factors by monocytes-macrophages, including IL-1β, IL-1ra (Vey et al, 1997) and TNF-α (Sampaio et al, 2000; Burger & Dayer, 2002). Additional experiments also detected release of MMPs from monocytes/THP-1 cells cultured with fixed T cells following activation with PHA, PMA and anti-CD3 (Lacraz et al, 1994). Production of TIMP has been detected from THP-1 cells when incubated with PHA/PMA activated, fixed T cells (Rezzonico et al, 1998).

Furthermore, Th1 and Th2 cells have different regulatory activities in cell-cell contact with THP-1 cells (monocyte cell line); Th1 cells induce higher level of pro-inflammatory cytokines such as IL-1β, whereas Th2 cells favours anti-inflammatory cytokines such as IL-1ra (Chizzolini et al, 1997). Moreover, direct contact between fixed PHA/PMA-activated T cells and fibroblast/synoviocytes inhibits the synthesis of type I and III collagen (Rezzonico et al, 1998). Cell-cell contact of T cells (Jurkat

cell line) with fibroblasts induced mRNA expression of IL-17 and IL-17 receptor (Thiele et al, 2000). Thus cell-cell contact represents an important mechanism for initiation of inflammation and tissue destruction. It has been suggested that cell-cell contact provides a mechanism that may participate in the process of inflammation. This has lead to identification of molecules on T cell surface that are involved in contact-dependent of monocyte-macrophage activation and cytokine production. For example it has been shown that activation of fibroblasts, synoviocytes and microvascular endothelial cells by stimulated peripheral blood T cells was mainly due to membrane-associated TNF-α and IL-1α (Burger et al, 1996; Malik et al, 1996; reviewed by Burger & Dayer, 2002). Monocyte/macrophage-T cell interactions obviously involve other surface molecules that have the ability to induce cell activation, for example CD40-CD40L, lymphocyte function associated antigen (LFA)-1/ICAM-1, and CD2/LFA-3. Ligation of CD40 (on the surface of the dendritic cells and macrophages) with CD40L (CD154, in T cells) regulates production of certain cytokines such as IL-8, MIP-1α, TNF-α, IL-12 and IL-1. Moreover, activation of human monocytes through CD40 induces MMP production (Malik et al, 1996; reviewed by Burger & Dayer, 2002; Brennan & Foey, 2002; Burger & Dayer, 2002). CD40 is expressed on a wide range of cells including monocytes and is involved in contact-dependent activation (Wagner et al, 1994; Yellin et al, 1995; Grewal & Flavell, 1998). CD40 and its ligand are responsible for interaction between T cells and B cells. This interaction leads to B cell proliferation and antibody production (Wagner et al, 1994). Intercellular adhesion molecule-1 (ICAM-1/CD54), a membrane glycoprotein, is a member of the immunoglobulin-like family with domains that play a role in cell-cell or cell-ECM adhesion interaction. ICAM is widely expressed on nonhaematopoietic cells such as vascular endothelial cells, and fibroblast and

haematopoietic cells such as monocytes, macrophages, T cells and dendritic cells. The interaction of ICAM-1 on the vascular endothelial cells with CD11a/CD18 (LFA-1) or CD11b/CD18 (Mac-1) on leukocytes enhances the adhesion of leukocytes to the vascular endothelium. Other surface molecules that have been implicated in T lymphocyte-monocyte interactions include CD69 and LFA-1 (Manie et al, 1993). It was shown that Jurkat T cells stimulated with anti-CD3, induced IL-1 production from THP-1 cells through cellular contact. LFA-1 was shown to be involved in this induction (Manie et al, 1993), and anti-CD69 inhibited IL-1 production, suggesting involvement of surface molecules in cytokine production (Isler et al, 1993).

1.7 The aim of the study

Proinflammatory cytokines play a central role in the progression of the inflammatory process in RA. Cytokine imbalance in favour of IL-1β over IL-1ra has been observed. However, the underlying mechanism controlling the production of IL-1β production remains unclear. There has been substantial interest in the induction of monocyte cytokine release following T cell-monocyte interactions. Moreover, T cells in the synovium of RA patients are under constant exposure to oxidative stress. This may alter the behaviour of T cells and their interaction with monocyte-macrophages.

Previous work done in our laboratory generated Jurkat T cells resistant to hydrogen peroxide (HJ16). These cells stimulate induction of proinflammatory cytokines such as IL-1β by differentiated THP-1 cells, compared to normal Jurkat cells (J16). The HJ16 cell line has now been cloned by using limiting dilution (1 cell/well), 35 clones were isolated. The primary aim of this study was therefore to characterize the HJ16 cells and the clones in the co-culture system using THP-1 cells or peripheral blood

mononuclear cells (PBMCs). Thus we can investigate whether oxidative stress alters the behaviour of the T cells (HJ16 and the clones) and their interaction with macrophages in relation to inflammatory joint disease.

It has been shown that Th1 and Th2 cells induce different cytokine patterns. It has been observed that Th1 cells induce IL-1β, whereas Th2 cells induce IL-1ra production upon cell-cell contact (Chizzolini et al, 1997), therefore, J16 and HJ16 cells might be classified into Th1 or Th2 phenotype using chemokine receptors specific for Th1 or Th2 cells.

Glutathione (GSH), depletion is associated with augmentation of oxidative stress-mediated proinflammatory cytokine production. Manipulation of anti-oxidants such as GSH in T cell lines (such as HJ16 and J16) and its effect on co-culture with a monocyte cell line such as THP-1 is investigated.

MAPK cascades are involved in inflammation and tissue destruction in RA. The use of MAP kinases inhibitors with T cell lines (J16 and HJ16) and their effects on the stimulation of IL-1β production by THP-1 cells are examined.

Reduced calcium signalling and low proliferative response in the rheumatoid synovial T cells are manifested. This reduction may be due to constant subjection to oxidative stress (Carruthers et al, 1996). Therefore calcium signalling and proliferation of J16 and HJ16, and the effects of GSH levels on calcium signalling and proliferation are investigated.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2 Materials and Methods

2.1 Materials

Antibodies

Mouse anti-human CD3 (OKT3) and mouse anti-human CD69 hybridomas were obtained from the American Type Culture Collection (ATCC), some anti-human-CD3 antibody was purchased from Biocarta (Oxford, UK) and from R &D Systems (UK). Mouse-anti-human-CD2 antibody was purchased from CYMBUS (UK). Purified mouse anti-human CD14 antibody was a kind gift from Dr D.M. Sansom. Goat anti-mouse IgG-FITC, and ELISA antibodies, IL-1β and IL-1ra were purchased from R&D Systems. Anti-CD28 antibody, anti-phosphorylated p38, and rabbit anti-horseradish peroxidase were kindly provided by Dr S.G. Ward (University of Bath).

Cell culture, ELISA and calcium signalling

Ionomycin, phorbol dibutyrate (PDBu), phytohaemagglutinin-L (PHA-L), lipopolysacchride (LPS, *Escherichia coli*), 1,25 dihydroxyvitamin D_3, (1,25 (OH) $_2$ D_3), glutaraldehyde, foetal calf serum (FCS), bovine serum albumin (BSA), o-phenylenediamine dihydrochloride (OPD) tablets, Tween-20, buthionine-DL-sulfoximine (BSO), N-acetylcysteine (NAC), diamide, diethyl maleate (DEM), sodium 3'-[1-[(phenylamino)-carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene-sulfonic acid hydrate (XTT), phenazine methosulfate, trichloroacetic acid (TCA), 5,5'-dithiobis (2-nitrobenzoic acid, DTNB), and β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (β-NADPH) were purchased from Sigma Chemical Company Ltd (Poole, Dorset).

Streptavidin-horseradish peroxidase was purchased from Serotec. Lymphoprep (for PBMC separation) was obtained from NYCOMED, Norway. RPMI 1640, sodium bicarbonate, pencillin/streptomycin, L-glutamine, Hanks balanced salt solution (HBSS) were purchased from GIBCO BRL.

PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor II), bisindolylmaleimide (protein kinase C inhibitor), fura-2 acetoxymethylester (fura-2 AM), glutathione reductase were purchased from CalBiochem.

The human cell line THP-1 was obtained from European Collection of Animal Cell Culture (ECACC). The human T-lymphocyte cell line Jurkat (J16) was obtained from the ATCC. Hydrogen peroxide tolerant Jurkat cell line was generated in our department. UV-A resistant Jurkat cell line was provided by Dr C. Pourzand, (University of Bath, Department of Pharmacy and Pharmacology).

Molecular Biology

CCR2, CCR3, CCR4 CCR5, CXCR3 and β-actin primers (forward and reverse primer), agarose and ethidium bromide were kindly provided by Dr Pauline Wood (Bath University, Department of Pharmacy and Pharmacology).

CCR2 sense primer sequence: CCAACTCCTGCCTCCGCTCTA,
Antisense primer: CCGCCAAAATAACCGATGTGATAC
CCR3, sense primer sequence: TGGCGGTGTTTTTCATTTTC,
Anti-sense primer: CCGGCTCTGCTGTGGAT.
CCR4: sense primer sequence GAAGAAGAACAAGGCGGTGAAGAT
Anti-sense sequence ATGGTGGACTGCGTGTAAGATGAG
CCR5 sense primer sequence TGCTACTCGGGAATCATAAAAACT
Anti-sense sequence TTCTGAACTTCTCCCCGACAAA

CXCR3 sense primer sequence CTCCACCTAGCTGTAGCAGA

Anti-sense sequence AGGAAGATGAAGTCTGGGAG

β-ACTIN sense primer sequence CTTTTCCAGCCTTCCTCC

Anti-sense sequence GCAGTAATCTCCTTCTGCATC

RNAZol was obtained from TEL-INC., PCR buffer (10x) with Mg^{2+}, deoxynucleoside triphosphate set (dATP, dCTP, dGTP and dTTP), and RNAsin were purchased from Roche Diagnostic. Poly $(T)_{12-18}$ were purchased from Amersham Pharmacia Biotech Inc.. 100-base pair DNA ladder, DNAse reaction buffer, reverse transcriptase (Superscript II) and DNAse 1 amplification grade were purchased from GIBCO, BRL.

General equipment

Tissue culture plastics were purchased from Fahrenheit Laboratory Supplies Ltd. Cell handling was performed in a laminar flow hood (Class II) and incubations were carried out in a 5% CO_2 at 37°C in a humidified incubator (Gallenkamp). Polypropylene tubes and polystyrene flasks were used for all cell maintenance and culture.

Cells were counted using a cell counter (Beckman coulter counter). FACS analysis was performed on a Becton Dickinson FACStar Plus, using a 100mW, 488nm argon laser, with light being channelled by 520nm filter (FL-1) and 580nm filter (FL-2). ELISA plate readings were performed on a Dynatech MR5000 spectrophotometer. Calcium signalling was performed on a fluorimeter (Photo-Technology International, PTI). The experiments under hypoxic conditions were performed using Anaerobic System incubators (USA), the oxygen tension was set and maintained constantly at

1% or 2% (14mmHg) by automatically injecting mixture of 1% or 2% oxygen and nitrogen into the chamber.

Some of the molecular techniques were carried out in the PCR hood (PCR-640, SafeLab). RT-PCR was performed using a thermocycler, (PCR system 2400, Perkin Elmer).

2.2 Methods

2.2.1 Tissue culture

The promonocytic cell line (THP-1), Jurkat T-cells line (J16), hydrogen peroxide tolerant cell line (HJ16), UV-A resistant Jurkat cell line (UVAR-J16) and clones of HJ16 (AB10, AB2, AD8, AF4, AF8, AG10, AH8, BD3, AD11, BB3, BG10 and BA6) were maintained in RPMI 1640 medium supplemented with foetal calf serum (see appendix) (at 0.3 x 10^6 –0.5x10^6 cells/ml) and cultured in 5% CO_2 at 37°C. This standard culture medium is referred to throughout this thesis as RPMI. All cell manipulations were performed under sterile conditions in a tissue culture laminar flow hood (Class II).

2.2.2 FACS analysis of H_2O_2 tolerance

To determine the resistance of cells (J16, HJ16 and HJ16 clones) to H_2O_2, cells were washed once in PBS, centrifuged for 5 minutes at 1600 rpm and maintained at 1 x 10^6 cells/ml. Cells were then transferred into polystyrene FACS tubes, and then washed again with PBS and resuspended with 1 ml of various concentration of H_2O_2 (0-30mM). H_2O_2 were prepared with serum free RPMI 1640 medium to avoid any reaction with FCS. Cells were then incubated for 4 hours at 37°C, 5% CO_2, 100µl of

FCS was added to all tubes and incubated overnight at 37°C, 5% CO_2. After that, cells were washed twice with PBS and resuspended with 50 µl of propidium iodide (see appendix1). Finally cells were analysed with flow cytometry (FL-2), using % PI uptake as an indication of cell death (PI binds to DNA of non-viable cells).

2.2.3 T cell-monocyte interaction and cytokine production

2.2.3.1 THP-1 cells differentiation

In order to use THP-1 cells as a model for monocyte/macrophages they were differentiated with 1,25-dihydroxyvitamin D_3 (1,25 (OH) $_2$ D_3). THP-1 cells were washed once with PBS and resuspended at 0.5 x 10^6 cells/ml in RPMI in a tissue culture flask. 1,25 (OH) $_2$ D_3 was added at a final concentration of 1 x 10^{-8}M in the dark and incubated at 37°C, 5% CO_2 for 48 hours. Differentiation was confirmed by using CD14 expression (detected with anti-CD14) as a marker for mature monocytes.

2.2.3.2 T cell line activation

J16, HJ16, UVAR-J16 and HJ16 clones were washed once with PBS and resuspended at 1 x 10^6 cells /ml in RPMI. Cells were activated with phorbol dibutyrate (PDBu) (0.5 ng/ml) and ionomycin (1µM), anti-CD3 (1 or 10µg/ml), anti-CD28 (10µg/ml) or PHA-L (10µg/ml). Cells were incubated at 37°C, 5% CO_2 for 24 hours. Activation was determined by monitoring the up-regulation of CD69 expression.

In some experiments, J16/HJ16 cells were pre-treated with BSO, DEM, diamide or NAC, to manipulate intracellular glutathione levels (see section 2.2.6 for detail). Incubation of mitogen activated protein kinase (p38, JNK, ERK1/2) or protein kinase C inhibitors were also used with J16 and HJ16 cells in some experiments prior to

activation. Cells were pre-incubated with 20μM PD98059 (ERK1/2 inhibitor), 1μM SB203580 (p38 inhibitor), 20μM SP600125 (JNK inhibitor II), 500nM bisindolylmaleimide (protein kinase C inhibitor), for 1 hour, and then washed once with PBS, resuspended in RPMI, and activated with various stimulants as mentioned above.

2.2.3.3 Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was kindly obtained from normal donors at Bath University, Department of Pharmacy and Pharmacology; 50-100 ml of blood were taken from each donor. Blood was diluted at 1:2 with PBS and layered carefully on top of 15 ml Lymphoprep in polypropylene tubes. Tubes were then centrifuged at 1600 rpm for 30 minutes. The middle layer was removed into new polypropylene tubes. PBMCs were then washed three times with RPMI (first wash was for 10 minutes and the further washes for 5 minutes, at 1600 rpm).

2.2.3.4 Enrichment of monocytes in PBMCs

PBMC were depleted of T/B cells in some experiments in order to determine if donor T/B cells in the co-culture of PBMCs interfered with J16, HJ16 and HJ16 clones. PBMCs were suspended at 5-10 x 10^6 cells/ml and were then centrifuged at 1600 rpm for 5 min. Supernatant was removed and the cells were resuspended with 500μl of mouse anti-human CD3 (T cell marker), and 100μl of mouse anti-human CD20 (B cell marker). Cells were rotated at 4° C for 1 hour and then washed twice with RPMI 1640 medium for 5 minutes at 1600 rpm. The pellet was resuspended in 1 ml of RPMI 1640 medium and 50 μl of magnetic DYNA beads (coated with sheep anti-mouse IgG) was added. The tube was rotated again at 4° C for 1 hour. 10 ml of RPMI 1640

medium was added to the tube, which was then placed horizontally on a magnetic bed for 2-3 minute at room temperature. This step was repeated three times. The non-adherent cells were recovered, and then analysed by using anti-CD3 for T cells (expression of CD3, as T cell marker) and anti-CD14 (expression of CD14 on the mature monocytes) (section 2.2.3.5).

2.2.3.5 Determination of cell surface marker expression

Activated and unactivated J16, HJ16, UVAR-J16, HJ16 clones and THP-1 cells were washed once with PBS and maintained at 0.5 x 10^6 cells/ml. Cells were transferred into FACS tubes and washed once with PBS for 5 minutes at 1600 rpm and resuspended with appropriate antibody (anti-CD14 for THP-1 cells differentiation, and anti-CD69 for J16, HJ16, UVAR-J16 and HJ16 clones activation). All tubes were incubated at 4°C for 30 minutes. Cells were then washed once with PBS for 5 minutes at 1600 rpm and resuspended with 50µl goat anti-mouse IgG antibody and incubated for 30 minutes at 4°C. Cells were analysed by flow cytometry (FL-1).

2.2.3.6 T-cell / monocyte co-culture technique

Co-culture experiments were carried out in 96 well U-shaped bottomed plates. All treatments were carried out in duplicate. THP-1 cells were used at 1 x 10^6 cells/ml, T cell lines were used at 5 x 10^6 cells/ml and PBMCs were co-cultured at 2 x 10^6 cells/ml. All cells were washed once and maintained at the appropriate concentration. In some experiments, J16, HJ16, UVAR-J16 cells and HJ16 clones (AB10, AD8, BA6, AG10, AF4, BB3 and BD3) were fixed with 0.025% glutaraldehyde for 5-10 minutes at room temperature. Aliquots (100µl) of J16, HJ16, UVAR-J16 and HJ16 clones suspension (activated or un-activated, fixed or unfixed) were added to 100 µl

differentiated THP-1 cells/PBMC/purified monocyte suspension in 96 well plates. Plates were incubated for 48 hours at 37°C, after which they were centrifuged and supernatants were harvested into new flat-bottomed 96 well plates.

2.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was performed to determine the production of IL-1β and IL-1ra in the supernatant of the co-cultures. ELISA plates (Nunc Maxisorb II Immunoplate) were coated with 50 µl/well of monoclonal primary antibody for IL-1β (mouse anti-human IL-β IgG, 17.5µg/ml) or IL-1ra (mouse anti-human IL-1ra IgG, 60µg/ml) diluted in 0.5 M carbonate/ bicarbonate buffer pH 9.6. Plates were sealed and incubated overnight at 4°C. Next day the plates were washed once with PBS-Tween (see appendix 1) and then blocked for 90 minutes with PBS containing 1% bovine serum albumin (BSA) and 5% sucrose. Plates were then washed 3 times with PBS-Tween and then either used immediately or sealed and stored at 4°C for up to one week.

Six standards for IL-1β (2000, 1000, 500, 200, 50, 0 pg/ml) and IL-1ra (10000, 5000, 2500, 1250, 625, 0 pg/ml) were prepared to generate standard curves. 50 µl/well of each standard were added into the first top row of ELISA plates. Culture supernatant samples were diluted 1:2 directly into ELISA plates with RPMI 1640 medium (25µl supernatant sample+25µl of RPMI 1640 medium). Plates were sealed and incubated overnight at 4°C. Plates were then washed 3 times with PBS-Tween. Secondary antibody (50µl/well) specific for anti-IL-1ra or IL-1β (goat anti-human IL-1β; goat anti-human IL-1ra) was added to the plates and incubated for 2 hours at room temperature. Plates were then washed 3 times with PBS-Tween and streptavidin-horse radish peroxidase (HRP) was added (50µl/well) and plates incubated for 30 minutes at

room temperature. After that, the plates were washed 4 times with PBS-Tween and dried thoroughly. OPD (o-phenylenediamine dihydrochloride) tablets were prepared in Milli-Ro water and added to the plates (50µl/well). Plates were incubated at room temperature for 30 minutes. Finally, 0.5 M sulphuric acid (50µl/well) was added to stop the reaction, and colour development was measured spectrophotometrically on a plate reader (absorbance at 490 nm).

2.2.5 Analysis of chemokine receptor mRNA

Expression of chemokine receptor mRNA for CCR5, CCR3, CXCR3 and CCR4 were analysed by using reverse transcriptase polymerase chain reaction (RT-PCR) to help characterize the nature of J16, HJ16 and HJ16 clones, as Th1 or Th2 like cells.

2.2.5.1 Extraction of mRNA

Messenger RNA was extracted from J16, HJ16 and HJ16 clones (AG10, AB2, BD3, AF4, AB10 AD8, BA6, AD11, BB3 and AF8). Cells (5-10 x 10^6 cells/ml) were washed once with PBS (5 minutes at 1600 rpm), resuspended with 1 ml RNAzol, and transferred into 1.5 ml Eppendorf tubes. 100 µl of chloroform was added to each tube. Tubes were shaken vigorously for 15 seconds, and then left on ice for 15 minutes. Tubes were centrifuged at 12000g for 15 minutes at 4°C and the RNA pellets were washed with 1 ml of 75% cold ethanol for 8 minutes at 7,500g at 4°C. Supernatant was removed and the pellet was air dried in the tissue culture hood, dissolved in RNase–free water and stored at -80°C.

2.2.5.2 DNAse treatment of RNA

mRNA was quantified using GeneQuant and the volume of RNA solution containing 1µg total RNA was determined. This volume was made up to 8µl with RNase–free water. 1µl of RNAzol (x10) reaction buffer was added and then 1µl of DNAse was added, the tube was mixed and left for 15 minutes at room temperature. 1µl of 25 mM EDTA was then added, and tubes were heated for 10 minutes at 65°C in the PCR thermal cycler.

2.2.5.3 Reverse transcription (RT)

Master mixture was prepared as follows: 4µl of 5x RT buffer, 1µl of DTT (dithiothreitol), 1µl of dNTP (deoxynucleoside triphosphate), 1µl RNAsin and 1µl reverse transcriptase (superscript II) (total volume 8µl which was sufficient for one sample). Tubes (one duplicate, labelled as RT+) containing the DNAse-treated RNA were placed on ice and 2µl of poly d (T) $_{12\text{-}18}$ was added to each tube. RNA samples (RT+) were then denatured in the PCR machine at 70°C for 10 minutes. 8µl of pre-prepared mixture was added to each tube and reverse transcribed by using the PCR machine at 42°C for 60 minutes, and at 95°C for 5 minutes and then held at 4°C. Samples were either stored at –20°C or carried on to the next step (PCR).

2.2.5.4 Polymerase Chain Reaction (PCR)

This step was carried out in the PCR hood. PCR master mixture was prepared as follows: 0.5µl of dNTP, 2.5µl of PCR buffer with Mg^{2+}, 0.125µl polymerase, 15.9 µl of RNase free water, 2.5µl forward/reverse primer of a chemokine receptor/ β–actin as control. This gives a total volume of 23.7 µl (for one sample).

23.7 µl of master mixture was added to new PCR tubes, labelled as RT+ or RT-. 1 µl of RT- sample or negative control (water) were added to appropriate tubes. The next step was carried out on the bench. 1 µl of RT+ samples (from section 2.2.5.3) were added to appropriate tubes. All tubes (RT+ and RT-) were placed in the PCR machine and the RT-PCR program was run, (95°C for 5 minutes, 56° C for 30 seconds over 35 cycles, 72°C for 30 seconds, 7 minutes at 72°C and held at 4°C).

2.2.5.5 Agarose gel electrophoresis of PCR products

Agarose gel was prepared by dissolving 5.6g of agarose in 300ml of 0.5x TBE (see appendix 1) (2% agarose in x0.5 TBE buffer) in a microwave. After cooling, 40µl/100 ml of ethidium bromide was added and the gel was made and left to solidify at room temperature. The electrophoresis tank was filled with 0.5x TBE buffer and the gel was placed into the tank. 5µl of blue juice (see appendix) was added to each sample. Samples were loaded into the gel and electrophoresis was carried out for 90 minutes, at 100 volts, at room temperature. Finally, the bands were detected with UV light.

2.2.6 Modulation of intracellular glutathione (GSH) levels in J16 and HJ16 cells

In order to deplete glutathione, cells (0.5 x 10^6 cells/ml) were incubated with various concentrations of buthionine-DL- sulfoximine (BSO), diamide or diethyl maleate (DEM), (see appendix for reagent preparation). BSO was used at 25, 50, 100 and 200 µM for 24 hours, diamide/DEM were used at 50 and 100 µM for 2 hours and 24 hours, or at 1mM for 2hours.

Increasing glutathione levels in J16 and HJ16 cells was achieved by incubating cells (0.5 x 10^6 cells/ml) for 24 hours with 2.5, 5, 10 mM N-acetylcysteine (NAC). Changes in levels of reduced glutathione were measured as described below (section 2.2.7).

All cells were washed three times with PBS for 5 minutes at 1500rpm, then resuspended with RPMI 1640 at 1x 10^6cells/ml and activated with ionomycin/ PDBu, co-cultured with THP-1 cells (section 2.2.3.6). Cells for calcium signalling were resuspended in HBSS, at 10-15x10^6cells/ml (section 2.2.8).

2.2.6.1 Measurement of intracellular GSH

Intracellular GSH was measured in J16 and HJ16 cells before and after treatment with BSO/NAC. The intracellular GSH levels were measured spectrophotometrically (see appendix1 for preparation of reagents): Cells were collected and washed in ice-cold PBS, and then resuspended in 5% TCA and kept on ice. Mixtures of 1ml phosphate buffer (see appendix1), 100 µl cell suspension, 50µl NADPH, 20 µl DTNB were prepared, and kept in a water bath at 25°C for 10 minutes. GSH reductase (100µl) was added to the mixture and absorbance was read immediately using spectrophotometer at 412 nm. A standard curve was constructed, and intracellular GSH concentration was determined (per 1x10^6cells).

2.2.7 Intracellular calcium signalling

Intracellular Ca^{2+} signalling was measured in J16, HJ16 and UVAR-J16 before and after treatment with BSO, diamide, DEM and NAC (see section 2.2.6). Cells were washed once with PBS, resuspended in RPMI at $1x10^7$ cells/ml and loaded with 1µl/ml (from stock of 2.5mM in DMSO) fura2-acetoxymethylester (fura-2-AM) to give a final intracellular concentration of 1-10µM fura2. Cells were then incubated in the dark for 45 minutes at 37°C to allow dye entry. After that, cells were washed three times and resuspended at 2 $x10^6$cells/ml in HBSS containing 1% BSA and 100µM calcium chloride.

Two millilitres of resuspended cells were then placed into a glass cuvette and loaded into a PTI spectrofluorimeter. A small magnetic stirrer placed in the bottom of the cuvette prevented cell sedimentation. Software provided with the system was used to configure the system and to set up the dual-excitation experimental method required for fura2 loaded-cells (fura2 excitation wavelength was driven between 340nm, absorption maximum of the Ca^{2+}-fura complex and 380 nm, isobetic wavelength) and emission wavelength of 510nm.

A calibration value was generated by addition of 20µl 4mM digitonin (in DMSO) to obtain R_{max} (the ratio of fluorescence intensities of the dye in the Ca^{2+} bound and Ca^{2+} free states at saturating amounts of Ca^{2+}), followed by 20µl 0.5mM EGTA (Ca^{2+} chelator) for R_{min} (the ratio of fluorescence intensities of the dye in the Ca^{2+} bound and Ca^{2+} free states at limiting concentration of Ca^{2+}). Absolute intracellular Ca^{2+} concentrations were calculated by using the following equation: $[Ca^{2+}]_I$ = Kd (R-R_{min})/ (R_{max}-R)Sf_2/Sb_2, where Kd is the effective dissociation constant of fura-2

(240nM), Sf2/Sb2 is the ratio of the 510nm fluorescence intensity of fura-2-loaded cells at minimum and maximum saturating Ca^{2+} respectively.

An initial intracellular calcium value was recorded for 50 seconds to confirm stable baseline, after which either PHA-L (20μg/ml), anti-CD3 (5μg/ml), anti-CD28 (5μg/ml), H2O2 (50μM), or a low concentration of ionomycin (500nM) was added to fura2-loaded cells to induce a Ca^{2+} response. The trace was recorded for 400 seconds. Results were analysed using software provided with the system, which utilises the equation mentioned above.

2.2.8 Proliferation assay, XTT

XTT is a colorimetric assay of cell viability, activation and proliferation, based on the use of tetrazolium salt. Some studies evaluated the use of XTT in human tumour cells and demonstrated the ability of electron coupling agents to potentiate bioreduction of XTT, which yields a highly coloured, water-soluble formazan product (Roehm et al, 1991).

In this section, XTT was used with Jurkat cells to assess the differences in proliferation among three cell lines, normal Jurkat cells, hydrogen peroxide tolerant Jurkat cells (HJ16) and UV-A resistant Jurkat cells (UVAR-J16) and also to examine the effect of various stimulants such as anti-CD3, anti-CD28, PHA, ionomycin, PDBu, and both ionomycin/PDBu. In addition, the effect of BSO, DEM, diamide (low GSH level) and NAC (high GSH level) on the proliferation of these cells was investigated (see section 2.2.6).

Cells were washed in PBS, resuspended in fresh RPMI 1640 at 0.1 x 10^5 cells/ml, and activated with anti-CD3, anti-CD28 (10μg/ml), PHA (10μg/ml), ionomycin (1μM), PDBu, (0.5ng/ml) and both ionomycin/PDBu. Cells were plated into flat bottom 96 well plates (100μl/well). The plates were incubated for 48 hours, fresh XTT-PMS solution (see appendix 1), was added, and after 4 hours incubation, the colour development was read on a spectrophotometer at 450nm.

2.2.9 Western Blotting

Cells were pre-treated with BSO and NAC for 24 hours, as described in section 2.2.6, to analyse any effect of GSH levels on activation of MAP kinases (p38). Cells were then washed in PBS, resuspended in serum-free RPMI 1640, and incubated for 2 hours at 37°C, 5% CO_2. Cells were then activated with PDBu for 15 minutes, 30 minutes, 1, 2.5, 5, 10 and 24 hour/s. Cells were centrifuged and washed twice in serum-free medium, 250μl of lysis buffer (Appendix 1) was added, the tubes were vortexed and kept on ice for 15 minutes. After that, tubes were centrifuged at 1000 rpm for 10 minutes at 4°C, and supernatants were harvested into new Eppendorff tubes. An aliquot (5 μl) was reserved for protein assay (see section 2.2.10) equal volume of sample buffer (appendix 1) was added to lysed cells (harvested supernatant) and boiled for 5 minutes.

Equal concentrations of protein of each sample were loaded onto 10% SDS-polyacrylamide gels, proteins bands were electrophoretically transferred onto nitrocellulose and the membrane was washed once in TBS-T (appendix 1) and blocked overnight with 1% BSA in TBS buffer, containing 10% sodium azide at 4°C, after that the membrane was washed three times in TBS buffer and then blotted with a

specific antibody to phosphorylated threonine and tyrosine of p38 MAP kinase for 2 hours at room temperature. After three washes with TBS, rabbit anti-horseradish peroxidase antibody was added and incubated for 1 hour at room temperature. The membrane was then washed five times in TBS, and incubated in ECL reagent for 1 minute. The bands were visualised onto Kodak film.

2.2.10 Protein assay, the Bradford assay

A range of standard concentrations of BSA (0.1, 0.2, 0.5, 1.0, 2.0 mg/ml) was prepared in lysis buffer (appendix1). Samples were diluted 1:5 in lysis buffer, 5µl of diluted samples and standard were then pipetted into 500µl of diluted (1:5, in Milli-Ro water) Bradford reagent in Eppendorff tubes, 100 µl of the mixture was then pipetted, in duplicate, into a flat-bottomed 96 well plate, and left for 5 minutes at room temperature. Finally the plate was read on a spectrophotometer (MR5000, Dynatech) at 760nm. Software provided with the system was used to calculate protein concentration.

2.2.11 Statistical analysis

Results are expressed as means +/- standard error of the mean. Statistical significance was analysed using analysis of variance (ANOVA) and student t-test. A p-value less than 0.05 was considered statistically significant.

CHAPTER 3

RESULTS

CHARACTERISATION OF A HYDROGEN PEROXIDE TOLERANT T CELL LINE IN CO-CULTURE WITH MONOCYTES

Chapter 3 Results

3.1 Characterisation of hydrogen peroxide tolerant T cell line in co-culture with monocytes

A T cell line tolerant to hydrogen peroxide (HJ16) was generated by treating Jurkat cells (J16) with increasing concentrations of H_2O_2. The HJ16 cell line was cloned by using limiting dilution. The main aim of the experiments reported in this chapter was to characterize some of these clones and assess any differences among them in the co-culture system.

In co-culture, J16, HJ16 cells and clones of HJ16 were incubated with differentiated THP-1 cells or peripheral blood mononuclear cells (PBMCs) in the presence or absence of lipopolysaccharide (LPS). Pro-inflammatory (IL-1β) and anti-inflammatory (IL-1ra) cytokines released by differentiated THP-1 cells or PBMCs co-cultured with J16, HJ16 cells and HJ16 clones were measured.

It has been noted that Th1 cells induce IL-1β, whereas Th2 induce IL-1ra production (Chizzolini et al, 1997). Therefore, it was decided to classify J16/HJ16/HJ16 clones into Th1 or Th2 phenotype. This was performed by identifying chemokine receptor mRNA associated with the Th1 or Th2 phenotype.

3.1.1 FACS analysis of H_2O_2 tolerance

In order to assess the tolerance of HJ16 cells or the clones to H_2O_2, cells were challenged with various concentrations of H_2O_2 for 4 hours at 37°C. The cells were then cultured overnight and analysed by flow cytometry (FL-2), using % PI uptake as an indicator of

cell death. As shown in figure 3.1, HJ16 cells and the clones exhibited higher H_2O_2 tolerance compared to J16. The ID_{50} of J16 cells were approximately 100μM, whereas, this value was 1mM for HJ16 cells and some HJ16 clones (AB10 and BB3) and even higher (1-3mM) for some other HJ16 clones, such as BD3, BA6, AF8, AG10, AF4, AH8, BG10 and AD8. AD11 and AB2 showed less resistance to H_2O_2 (ID_{50} of 100-300μM), and so were discarded.

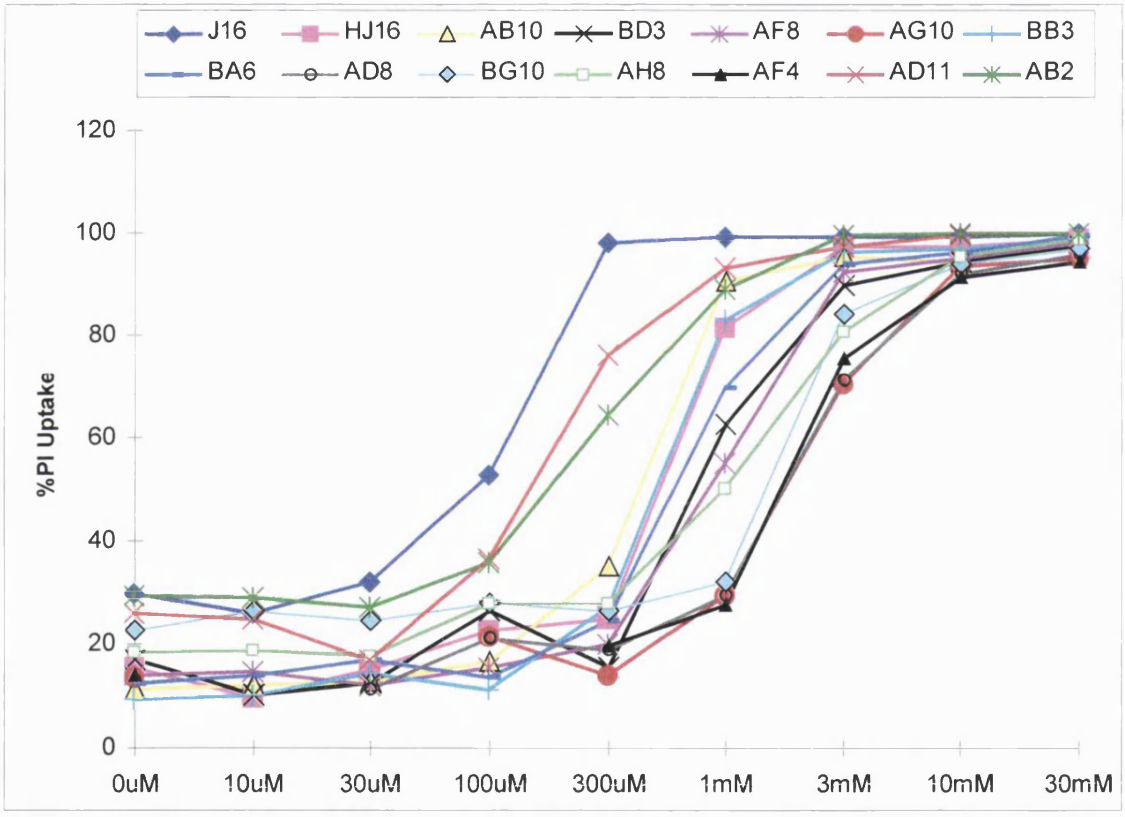


Figure (3.1): J16, HJ16 and HJ16 clones cell death following H_2O_2 challenge.
Death of J16, HJ16 and HJ16 clones (AB10, BD3, AF8, AG10, BB3, BA6, AD8, BG10, AH8, AF4, AD11, AB2), incubated with various concentrations of hydrogen peroxide (10μM-30mM) for 4 hours then washed and cultured overnight. Cell death was measured as increase in FL-2 fluorescence due to uptake of propidium iodide (PI) by necrotic cells, determined by flow cytometry.

3.1.2 Differentiation of THP-1 cells with 1,25-dihydroxyvitamin D_3

In order to obtain inducible IL-1β from the pro-monocytic cell line THP-1, these cells require differentiation to a mature form. This can be achieved by incubation of cells with 1,25-dihydroxyvitamin D_3 (1,25 $(OH)_2$ D_3) for 48 hours before use. Differentiation was determined by expression of CD14, a monocyte surface marker. This assay has been used routinely in all experiments to indicate differentiation of THP-1 cells prior to co-culture.

THP-1 cells were washed once in PBS, re-suspended at concentration of 0.5 x 10^6 cells/ml in RPMI, 1,25 $(OH)_2$ D_3 was added at final concentration of 10^{-8}M and incubated for 48 hours. The cells were then washed three times in PBS and re-suspended in RPMI. Cells were then analysed for CD14 surface expression using flow cytometry. The results below (Figure 3.2) are representative of routine testing. Differentiation of cells was exhibited as a shift in the fluorescence towards the right (figure 3.2b).

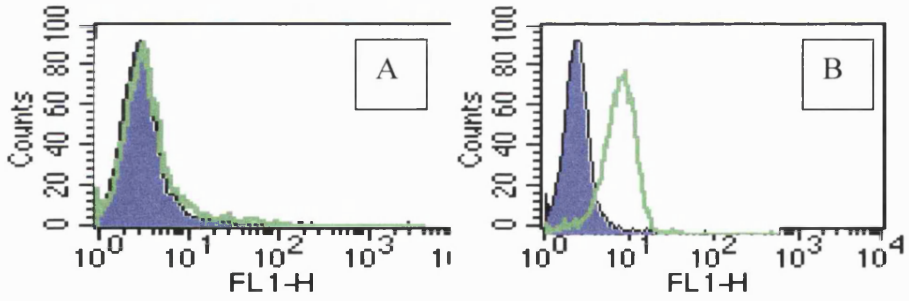


Figure (3.2): Determination of THP-1 differentiation by using anti-CD14 antibody. Determination of THP-1 cells differentiation due to 1,25$(OH)_2$ D_3 is measured as the level of FL-1 fluorescence due to binding of FITC conjugated secondary antibody, which binds to CD14 (specific primary antibody), detected by flow cytometry. Histograms of FL-1 fluorescence, (A) -1,25$(OH)_2$ D_3, (B) +1,25$(OH)_2$ D_3 are plotted. Background (filled histogram) = (–) anti-CD14 antibody, and opened histogram= (+) anti-CD14 antibody.

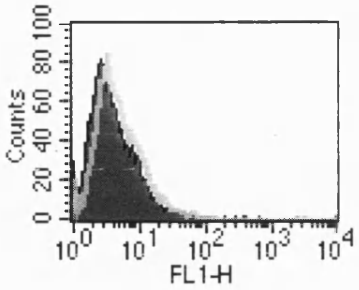
3.1.3 Activation of J16, HJ16, and HJ16 clones and surface marker expression

In the co-culture system, resting J16 and H16 cells have been shown to provide a limited stimulation to THP-1 cells or PBMCs, resulting in a small increase in IL-1β and IL-1ra production. In order to investigate possible methods of cell activation and the effect of this activation on induction of IL-1β and IL-1ra production, J16, HJ16 cells and HJ16 clones (AB10, AD8, BA6, BD3, AG10, BB3 and AF4) were activated with PBDu/ionomycin or PHA-L. This activation was measured by determining CD69 expression, one of the earliest markers of T cell activation, reaching a peak within 24 hour and declining slowly over the course of several days.

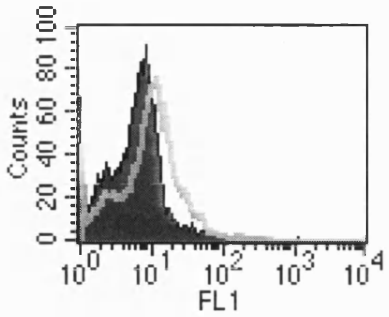
Under resting conditions, the HJ16 cells exhibited low but detectable expression of CD69, indicating that these cells are slightly activated at resting conditions, perhaps due to chronic exposure to H_2O_2. Ionomycin alone had a limited effect on CD69 expression on both J16 and HJ16 cells. PDBu and PHA-L (10-20μg/ml) produced high activation, as manifested by CD69 expression on both cell types, as displayed in Figure 3.3a/b.

Moreover, there was a difference in the responsiveness of J16 and HJ16 cells to PHA-L: HJ16 cells were more responsive to PHA-L (0.1-10 μg/ml). Stimulation with 0.5-10 μg/ml PHA-L resulted in a significant increase in both CD69 and CD2 expression compared to J16 cells. HJ16 cells responded at low concentration of PHA (0.1 μg/ml), whereas J16 cells required concentrations above 1μg/ml, as shown in figure 3.5. Both unactivated J16 and HJ16 cells express low level of CD2, but slightly higher with HJ16 cells, addition of PHA-L increased this expression, as shown in Figure 3.5b.

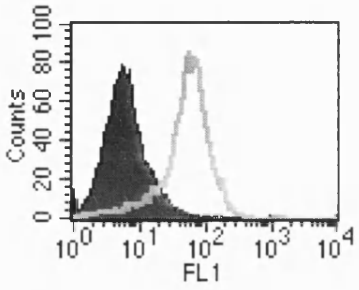
Activation with ionomycin and PDBu together has been used to characterise HJ16 clones in co-culture, and so CD69 expression was monitored in these cells as shown below (Figure 3.4). Like HJ16 cells, unstimulated HJ16 clones also exhibited low expression of CD69, indicating that these cells are slightly activated under resting conditions. No significant difference was observed in CD69 expression on activated J16/ HJ16 cells or HJ16 clones.



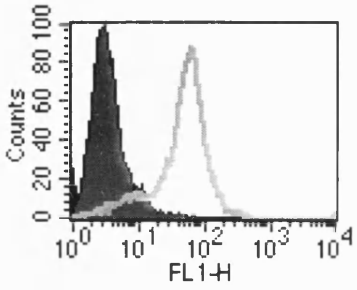
(A) Resting J16 (no CD69 expression)



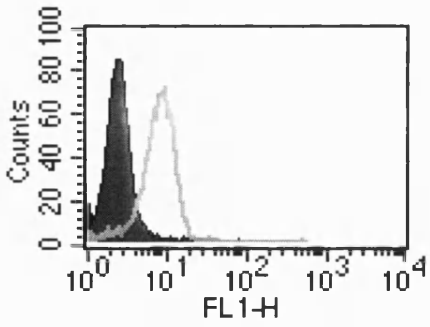
(B) Activated J16 with ionomycin (low CD69 expression)



C) Activated J16 with PDBu (high expression of CD69)

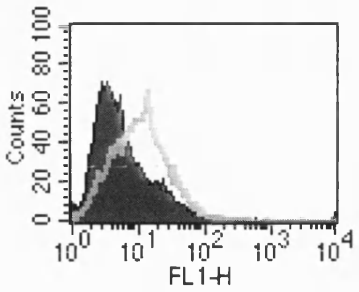


D) Activated J16 with PDBu/Ionomycin (high expression of CD69)

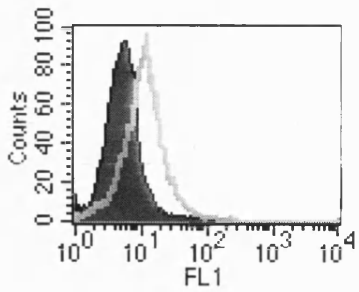


E) Activated J16 with PHA-L (high CD69 expression)

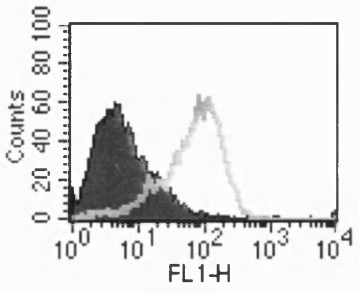
Fig (3.3a): Flow cytometry results (FL-1) of CD69 expression on J16 cells: Investigation into the effect of various stimulants on T-cell activation, J16 cells were activated with ionomycin, PDBu, both PDBu/ ionomycin and PHA-L (10-20 µg/ml). Resting J16 showed no activation (A). Very low activation was achieved with ionomycin alone (B), PDBu (C), ionomycin/PDBu (D) and PHA-L (E) activation resulted in high expression of CD69 (a shift in FL-1 fluorescence to the right, open histogram).



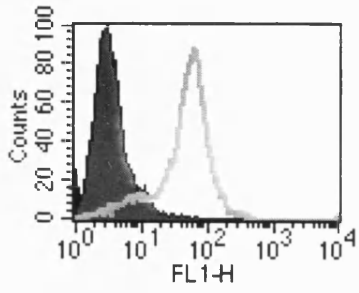
(A) Resting HJ16 (low CD69 expression)



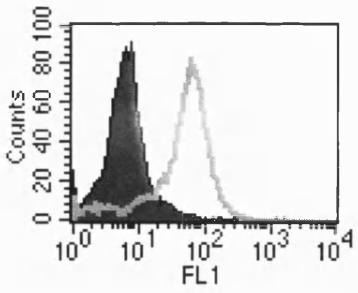
(B) Activated HJ16 with ionomycin (low CD69 expression)



C) Activated HJ16 with PDBu (high expression of CD69)

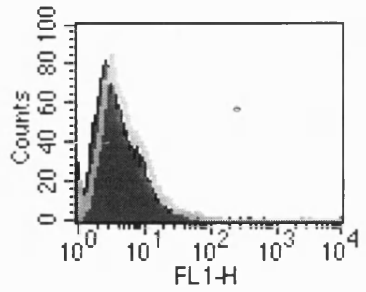


D) Activated HJ16 with PDBu/Ionomycin (high expression of CD69)

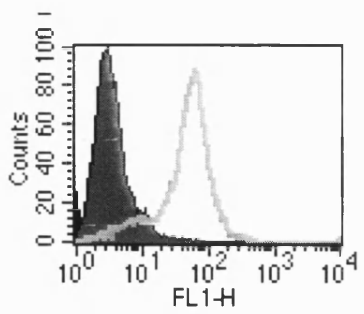


E) Activated HJ16 with PHA-L (high CD69 expression)

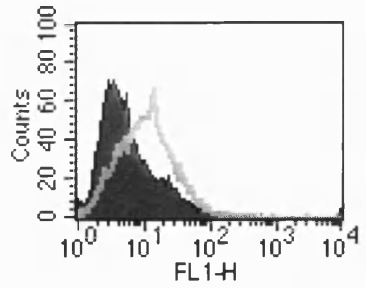
Fig (3.3b): Flow cytometry results (FL-1) of CD69 expression on HJ16 cells: Investigation into the effect of various stimulants on T-cell activation, HJ16 cells were activated with ionomycin, PDBu, both PDBu/ionomycin and PHA-L. Resting HJ16 cells were slightly activated (A). Very low activation was achieved with ionomycin alone (B). PDBu (C), ionomycin/PDBu (D) and PHA-L (E) activation produced high expression of CD69 (a shift in FL-1 fluorescence to the right, open histogram).



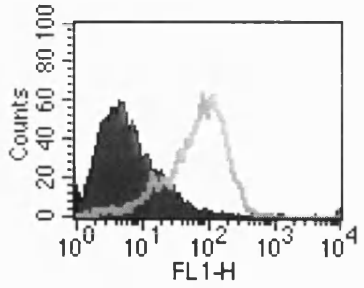
(A) Resting J16 (no expression of CD69)



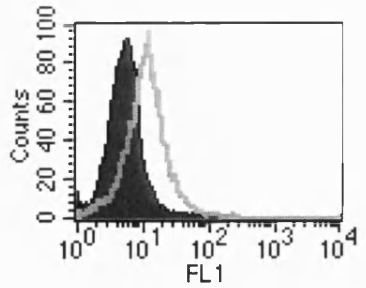
(B) Activated J16 (high expression of CD69)



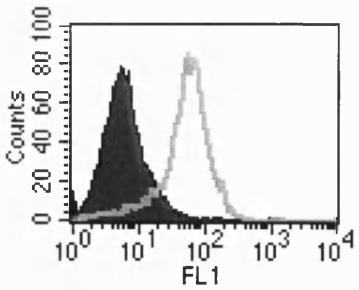
(C) Resting HJ16 (low expression of CD69)



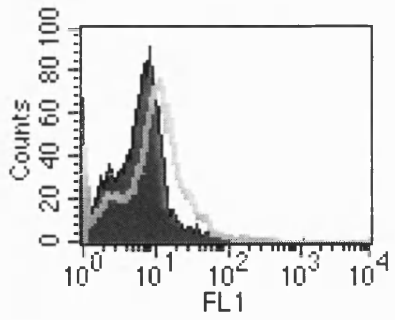
(D) Activated HJ16 (high Expression of CD69)



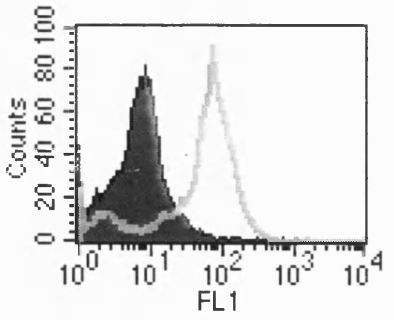
(E) Resting AB10 (low Expression of CD69)



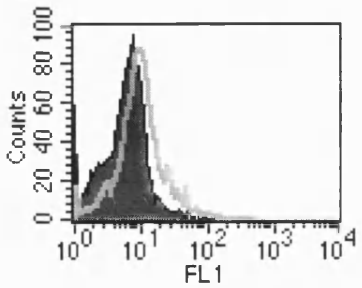
(F) Activated AB10 (high Expression of CD69)



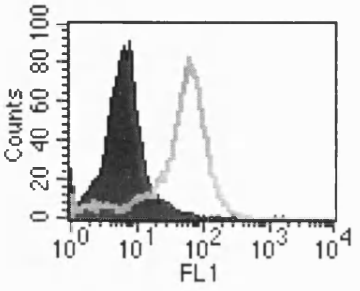
(G) Resting AD8 (low Expression of CD69)



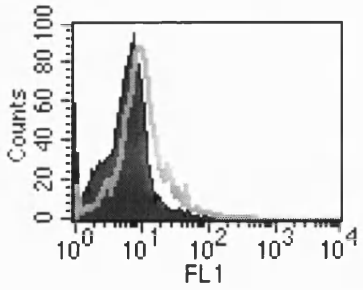
(H) Activated AD8 (high Expression of CD69)



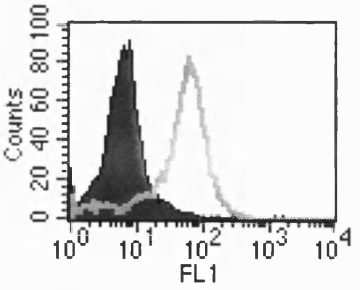
(I) Resting BA6 (low Expression of CD69)



(J) Activated BA6 (high Expression of CD69)



(K) Resting BD3 (low Expression of CD69)



(L) Activated BD3 (high Expression of CD69)

Figure (3.4): Flow cytometry results (FL-1) of CD69 expression on J16, HJ16 and clones of HJ16 (AB10, AD8, BA6 and BD3). HJ16 cells and clones showed slight activation at resting phase compared to J16 cells, which showed no activation (no expression of CD69). All activated cells (with PBDu /ionomycin or PHA) showed increased expression of CD69 (a shift in FL-1 fluorescence to the right, open histogram).

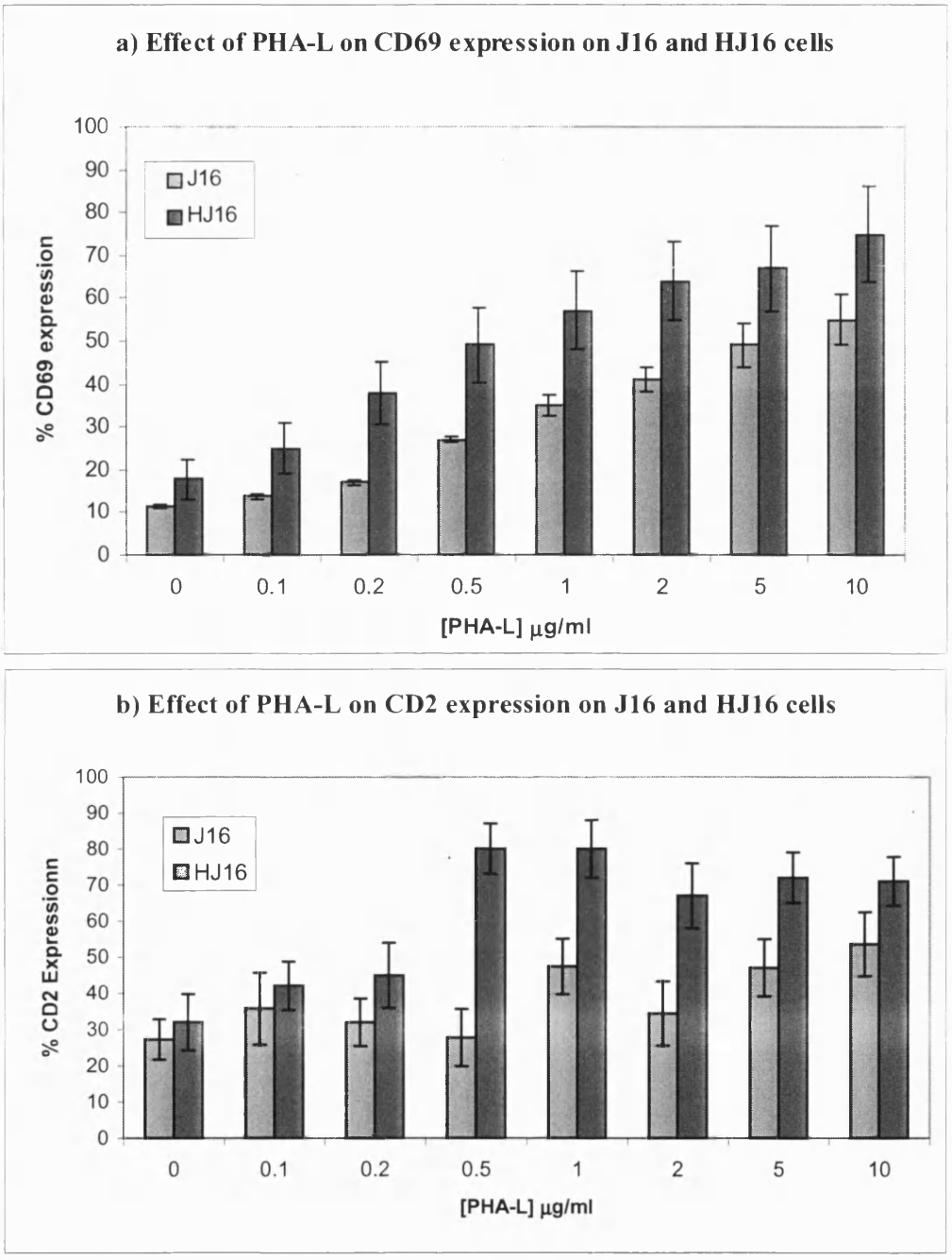


Figure 3.5 Effect of various concentration of PHA-L on the expression of (a) CD69 and (b) CD2 on J16 and HJ16 cells. A slightly higher expression of CD69 and CD2 was seen with resting HJ16 cells compared to resting J16 cells. Stimulation with PHA-L (0.1-10 µg/ml) resulted in a significant up-regulation of both CD69 and CD2.The data is the mean of five experiments, +/- standard error of the mean (SEM).

3.1.4 Expression of chemokine receptor mRNA

In this section, the expression of chemokine receptor mRNA associated with Th1/Th2 cells was determined in order to identify the phenotype of J16, HJ16 and the clones and also to examine whether the chronic exposure to H_2O_2 (oxidative stress) altered the Jurkat cells phenotype or had an effect on the expression of chemokine receptor at the mRNA level.

Chemokine receptor mRNA detection was performed for CCR3, CCR4 (Th2 chemokine receptors), CCR5 and CXCR3 (Th1 chemokine receptors) and CCR2 (common receptor for Th1 and Th2) by using RT-PCR. A positive result of mRNA was indicated as a clear band, and β-actin was used as positive control (Figure 3.6). CCR2, CCR3 and CCR5 mRNA appear as clear bands for all types of cells tested. However, CCR4 mRNA bands are slightly unclear for some cells such as HJ16 cells, although a clearer band for HJ16 CCR4 mRNA was obtained in some experiments. This may be due to technical problems, since the PCR technique is very sensitive and not quantitative. None of the cells expressed CXCR3 mRNA (Th1 marker), since no bands of CXCR3 mRNA were observed. The experiment was repeated five times for all chemokine receptors and very similar results were obtained each time.

No difference was observed between J16 and HJ16 cells or the clones in the expression of these chemokine receptor mRNA. CCR5 may not be a specific marker of Th1 cells, since it has been reported also as a Th2 chemokine receptor (reviewed by Sallusto; 1998). The pattern of chemokine mRNA species isolated implies that the J16 cells are perhaps more Th2-like than Th1-like, although different experiments would be needed to justify this conclusion.

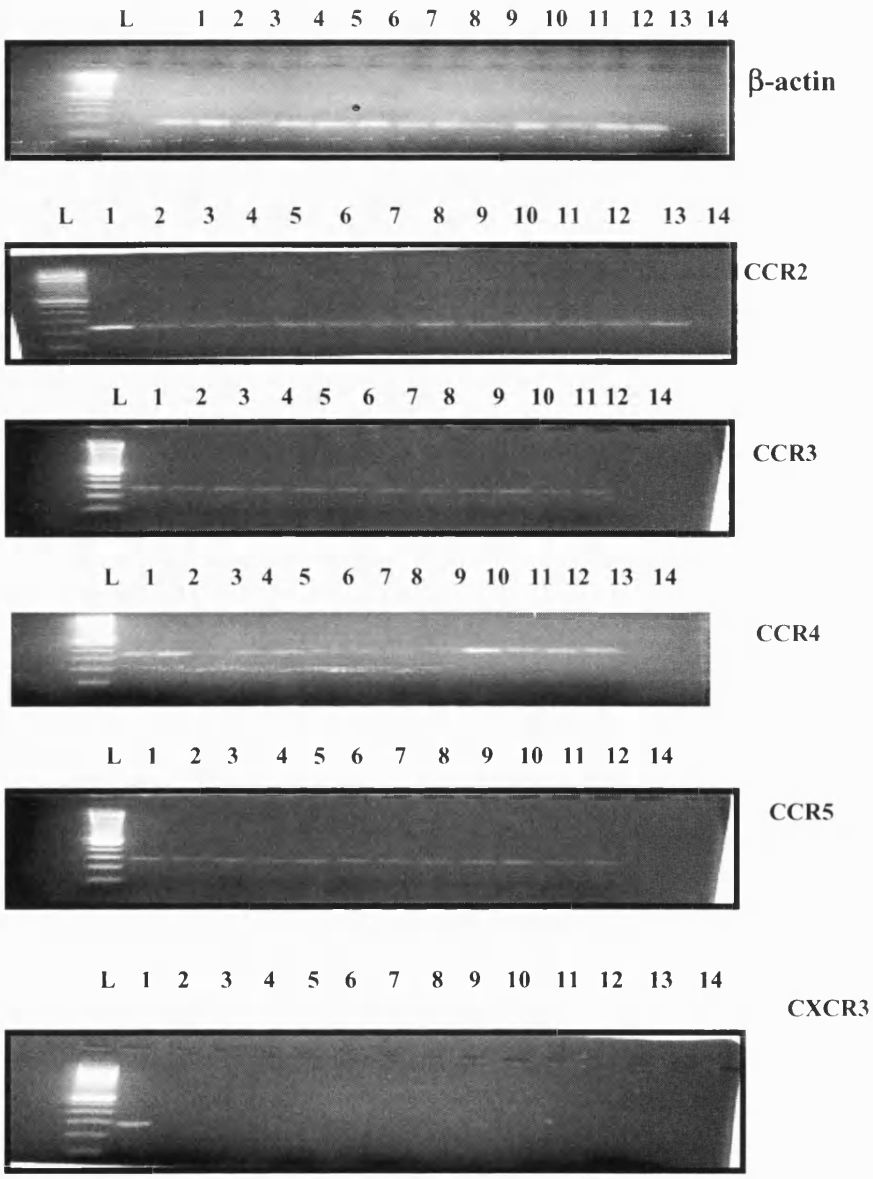


Figure (3.6): RT-PCR results for some chemokine receptors of Th1 and Th2. Results shown are a representative example of five similar results for each chemokine receptor.

L=DNA ladder, 1=PBMC (Positive control), 2=J16, 3=HJ16, 4=AB10, 5=AF4, 6=AG10, 7=AD8, 8=AH8, 9=AD11, 10=BA6, 11=BB3, 12=BD3, 13=AB2, 14=Negative control.

3.1.5 Cytokine production (IL-1β/IL-1ra) in co-culture

In order to investigate T cell / monocyte interactions, T cells such as J16, HJ16 cells and clones of HJ16 cells (AB10, AD8, BA6, BD3, AG10, AF4 and BB3) were cultured with PBMC or THP-1 cells. This co-culture technique reveals several interesting points on the effect of T cells on monocytes, as illustrated below.

3.1.5.1 IL-1β and IL-1ra production by PBMCs

J16, HJ16 cells and HJ16 clones (activated /un-activated, fixed or unfixed) were co-cultured with fresh PBMCs, (with/without LPS) for 48 hours at 37°C and 5% CO_2.

Low levels of IL-1β and IL-1ra were produced by control PBMCs in the absence of LPS; however, this amount was increased significantly ($p<0.01$) after addition of LPS. Un-activated cells (J16, HJ16 and HJ16 clones) had no significant effect on the production of either IL-1β (Figure3.7a) or IL-1ra (Figure 3.7c) by PBMCs in the presence or absence of LPS.

Activation of T cell lines (J16, HJ16, and HJ16 clones) by PDBu/ionomycin resulted in a significant stimulation of both IL-1β (Figure 3.7a) and IL-1ra (Figure3.7c) production by PBMCs compared to un-activated T cell lines ($p<0.01$). Activated unfixed HJ16 cells and HJ16 clones (AB10, AD8, BA6, AG10, AF4, BD3, and BB3) significantly induced higher levels of IL-1β compared to J16 cells in the presence of LPS ($p<0.05$). Unfixed activated HJ16, and HJ16 clones induced slightly more IL-1ra production by PBMCs than J16 cells.

A significant decrease in IL-1ra production by PBMCs was obtained with all fixed activated T cell lines. A slight stimulation of IL-1β and IL-1ra production by PBMCs was generated by fixed T cell lines (Figure 3.7b, d), indicating that soluble factors may be mediating the production of both cytokines by PBMCs.

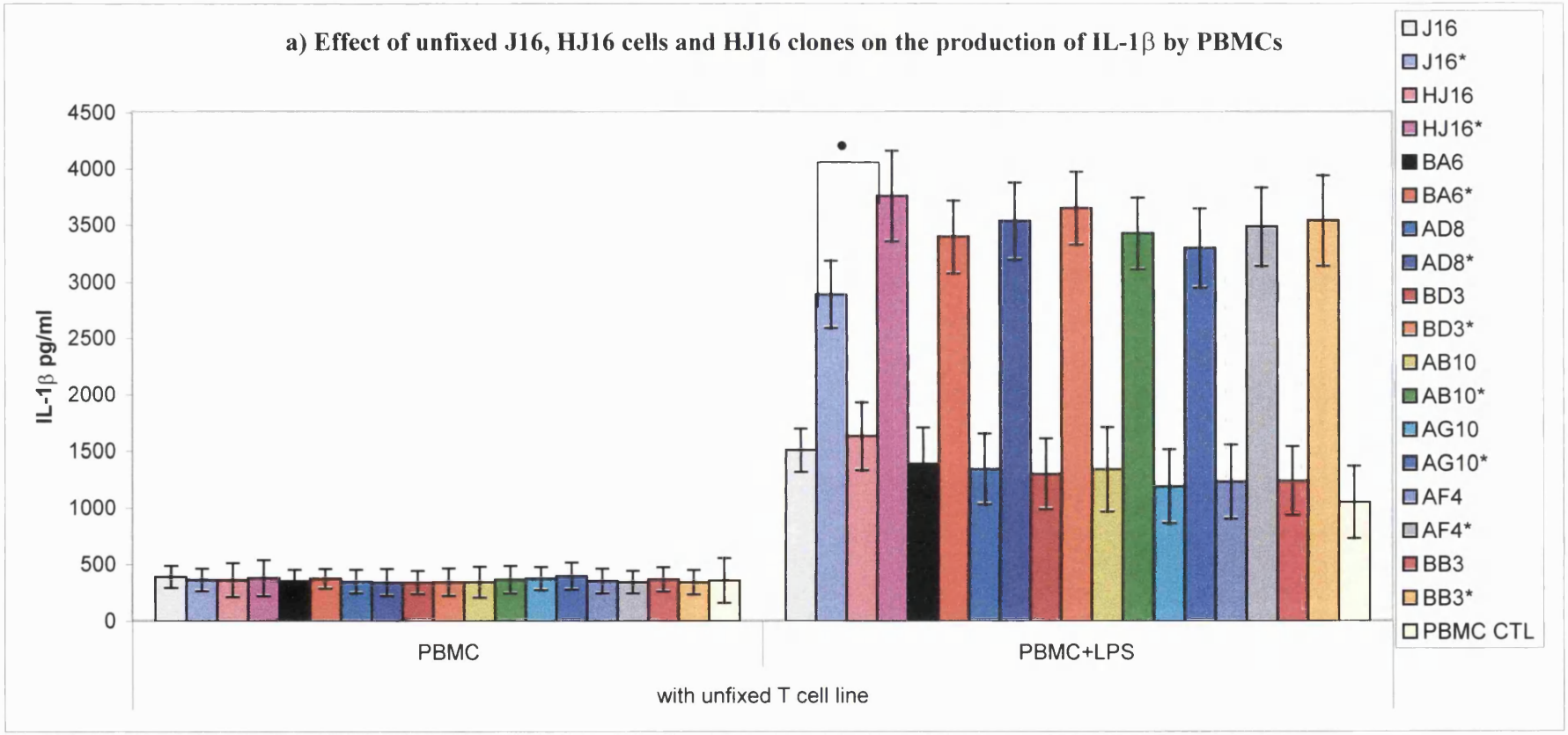


Figure (3.7a) ELISA results of IL-1β, unfixed J16, HJ16 and HJ16 clones were cultured with fresh PBMCs. Both HJv16 cells and the clones of HJ16 cells stimulated more IL-1β compared to J16 cells. Data shown are the mean of seven experiments (+/-SEM). Each experiment was done in duplicate, (*= Activated cells with PDBu and ionomycin, • indicates p value<0.05).

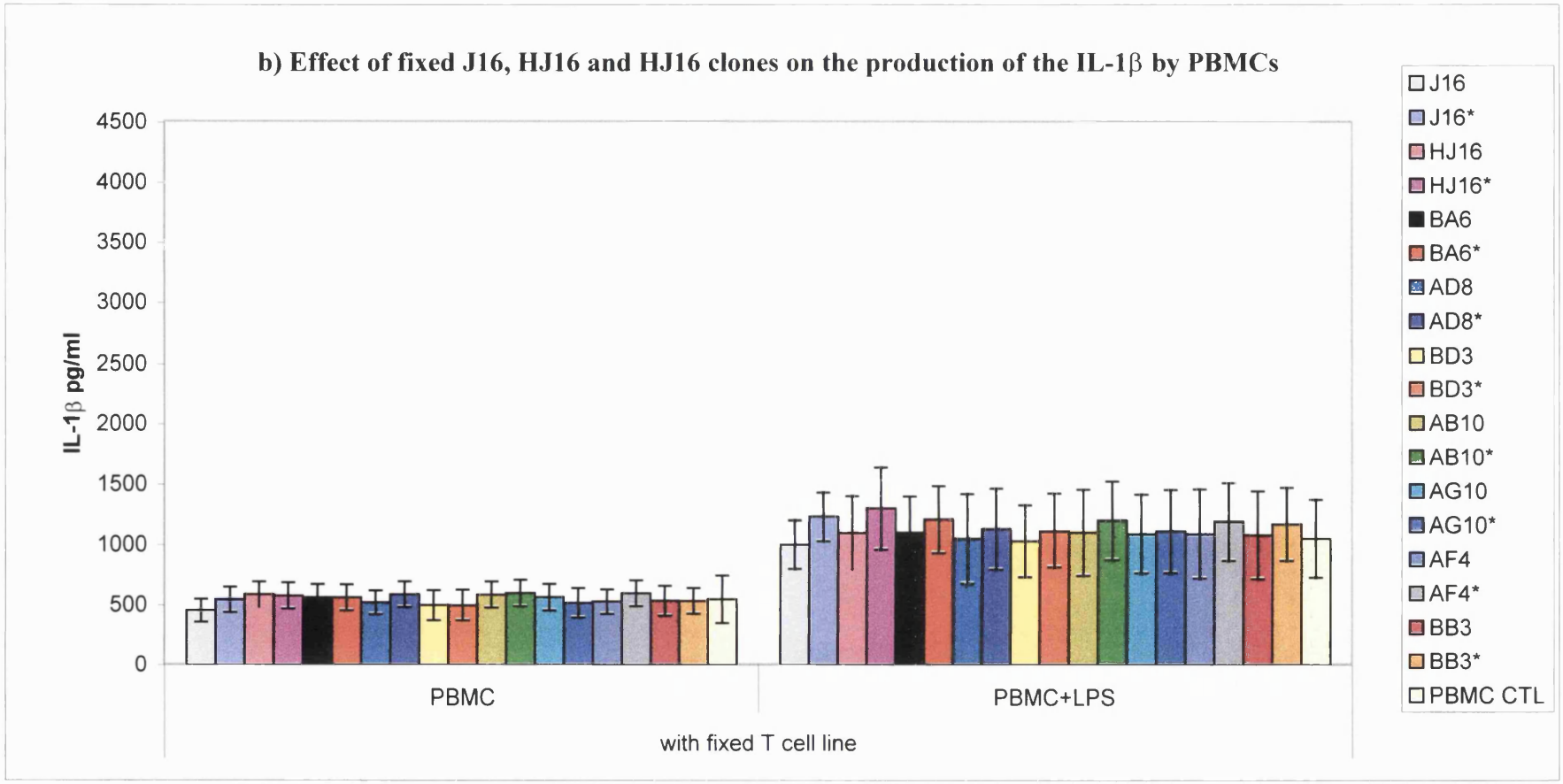


Figure (3.7b) ELISA results of IL-1β, fixed J16, HJ16 and HJ16 clones were cultured with fresh PBMCs. Fixed T cell lines failed to stimulate IL-1β production by PBMCs. Data shown are the mean of seven experiments (+/-SEM), (*= Activated cells with PDBu and ionomycin).

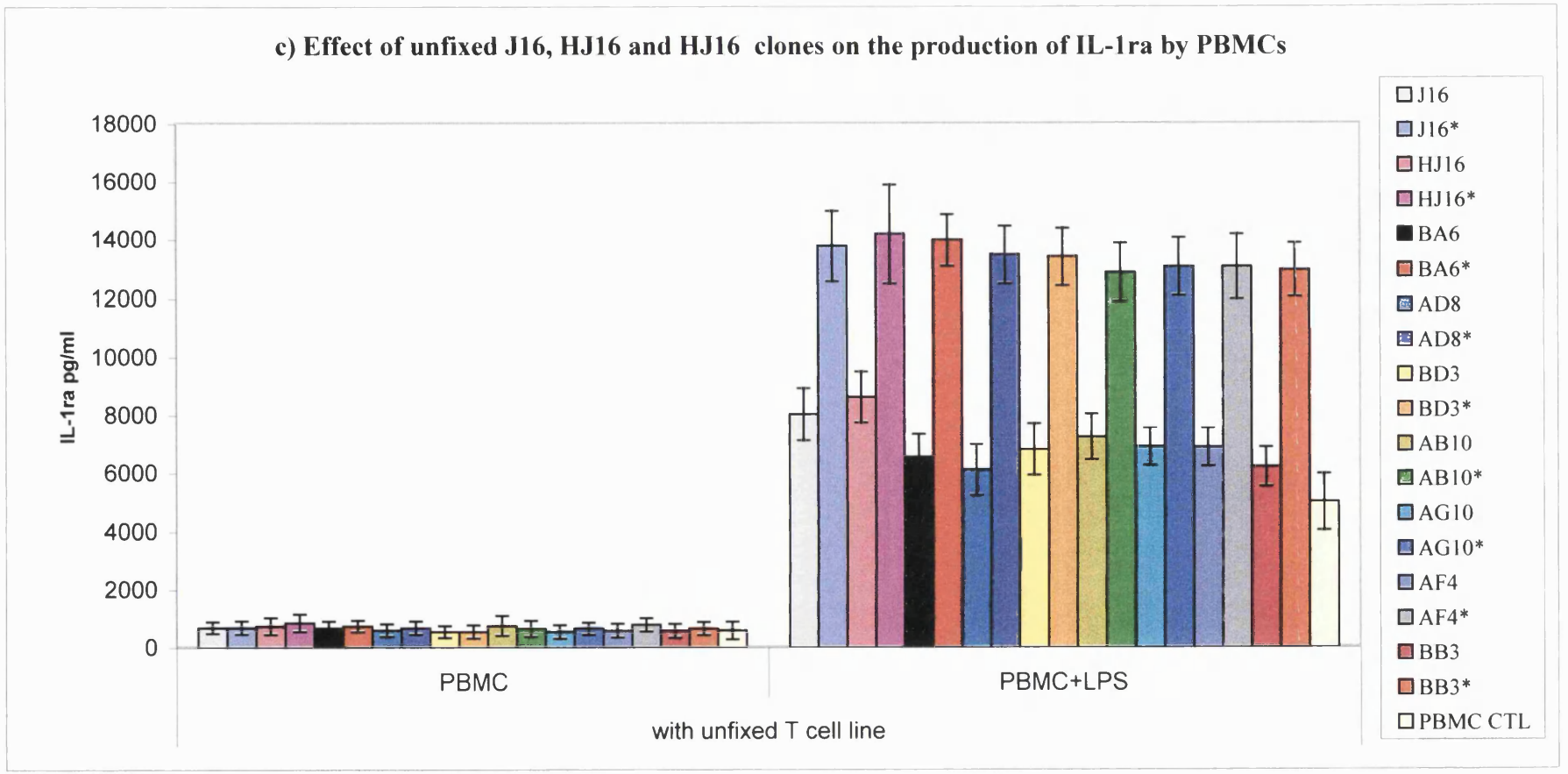


Figure (3.7c) ELISA results of IL-1ra, unfixed J16, HJ16 and HJ16 clones were cultured with fresh PBMCs. No significant differences in the stimulation of IL-1ra by J16, HJ16 cells and HJ16 clones. Data shown are the mean of seven experiments (+/-SEM), (*= Activated cells with PDBu and ionomycin).

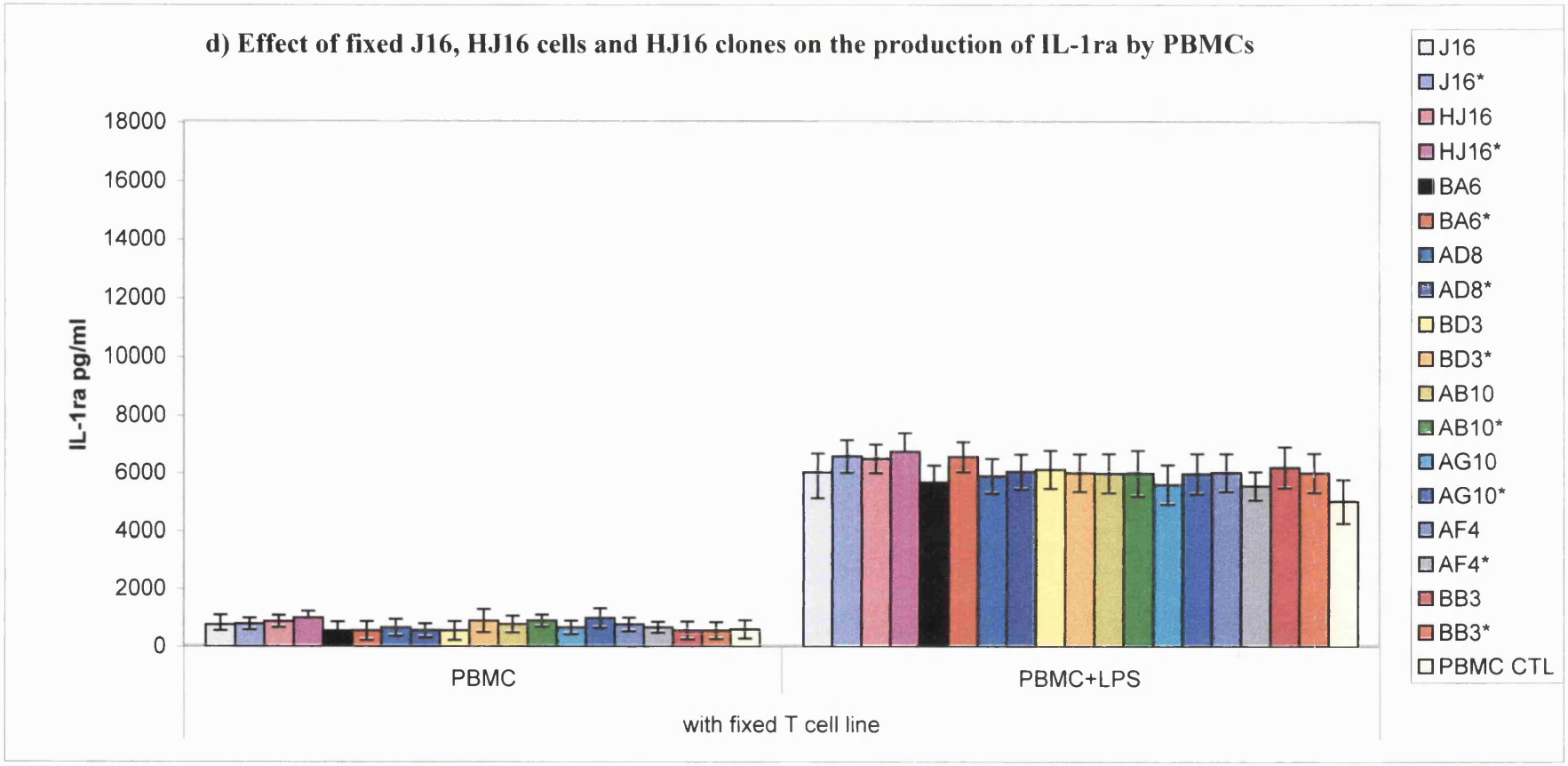


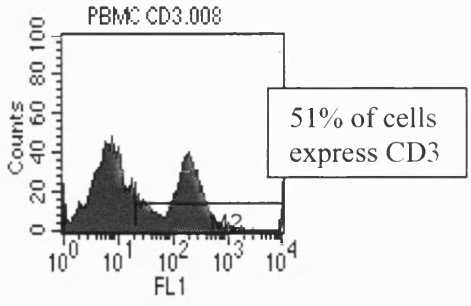
Figure (3.7d) ELISA results of IL-1ra, fixed J16, HJ16 and HJ16 clones were cultured with fresh PBMCs. Fixed J16, HJ16 cells and the clones of HJ16 cells failed to stimulate IL-1ra production by PBMCs. Data shown are the mean of seven experiments (+/- SEM), (*= Activated cells with PDBu and ionomycin).

3.1.5.2 Control experiments: Comparison between fresh PBMCs and purified monocytes in production of IL-1β and IL-1ra

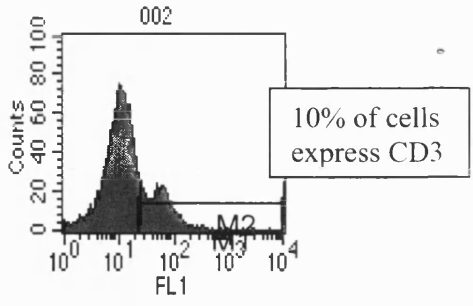
PBMCs were depleted of T cells (about 80-85 % of T cells were removed), and enriched with monocytes (express CD14). This was done to reduce any influence of donor T cells in the co-culture assay. Experiments showed a similar pattern of cytokine production by both PBMCs and enriched monocytes (depleted PBMCs of T cells and B cells). T and B cells were removed by negative selection employing anti CD3 (T cells marker) and anti-CD20 (B cell marker). This technique was partially successful, since % monocytes (CD14 expression) was increased from 9% (Figure 3.8, A2) in total PBMC to 68% (B2) in enriched monocytes. PBMCs showed 51% of CD3 positive cells expression (A1), whereas this % was decreased to10% (B1) after the treatment (Figure 3.8).

ELISA results of IL-1β and IL-1ra produced by both PBMCs and enriched monocytes are shown in Figure 3.9a,b. Similar patterns were obtained in both cultures, indicating that any influence of donor T cells in the stimulation of monocytes to produce cytokines is minimal. However, the amount of cytokines released by purified monocytes was slightly less than by PBMCs, which may be due to subjection of the cells to several purification steps. J16 and HJ16 did stimulate the production of both cytokines, and HJ16 cells were a better stimulant, in both experiments.

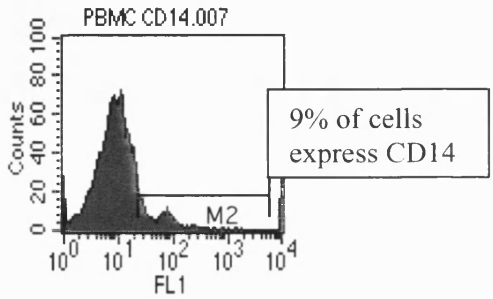
A1: PBMCs: CD3 expression



B1: Purified monocytes: CD3 expression



A2: PBMCs: CD14 Expression



B2: Purified monocytes: CD14 expression

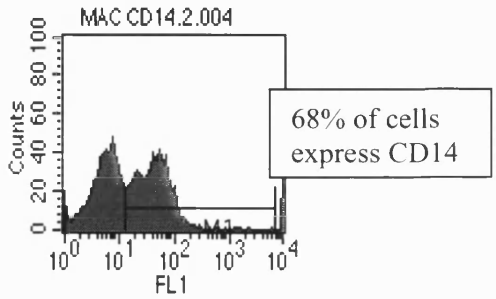


Figure (3.8) PBMCs were depleted of T cells and B cells (purified monocytes) and the expression of CD3 (T cell marker) and CD14 (monocyte cell marker) were analysed.

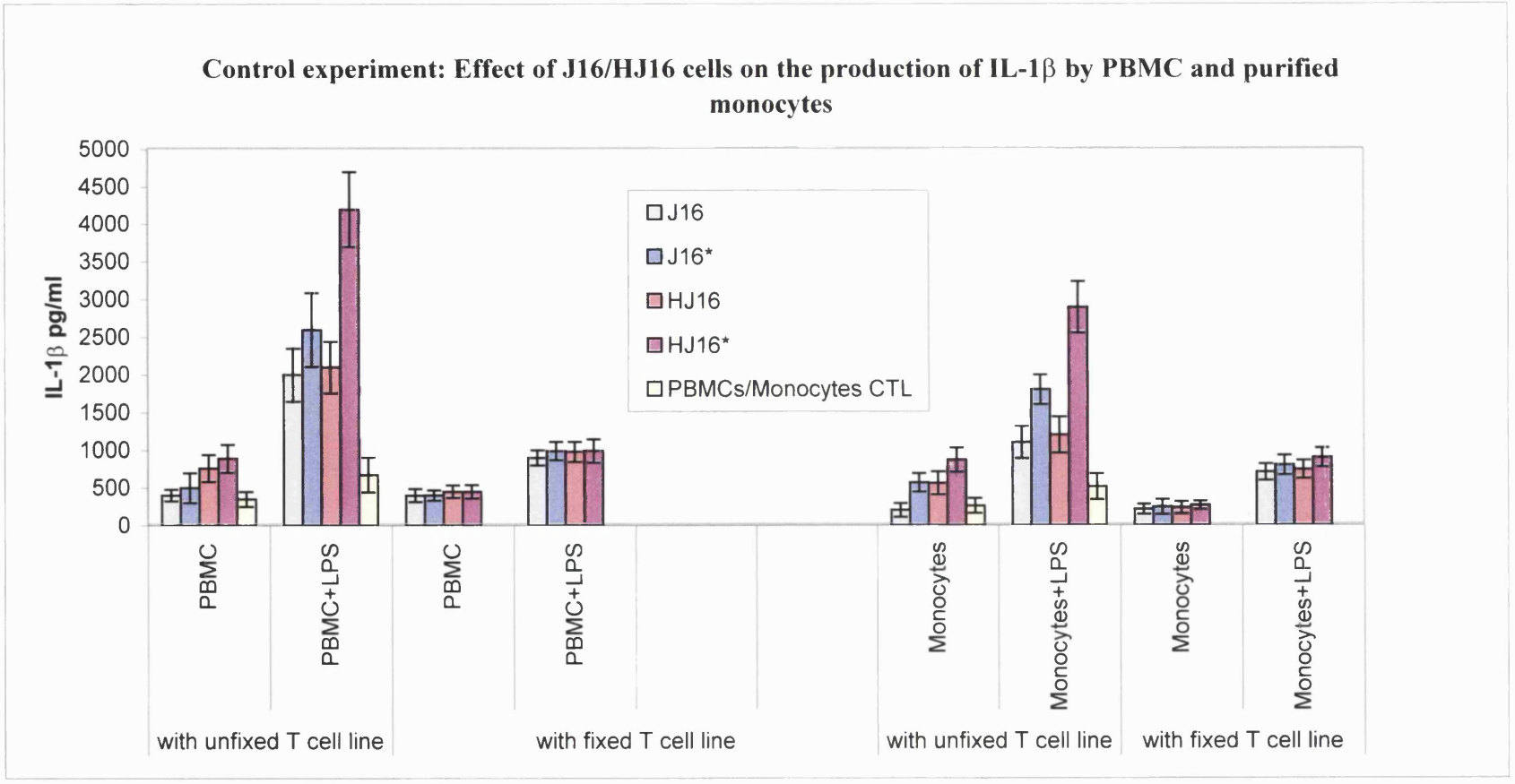


Figure (3.9a) ELISA results of PBMCs and monocytes, illustrates a similar pattern of IL-1β produced by PBMC and purified monocytes. (The result represents the mean of three experiments (+/- SEM), (*= Activated cells with PDBu and Ionomycin).

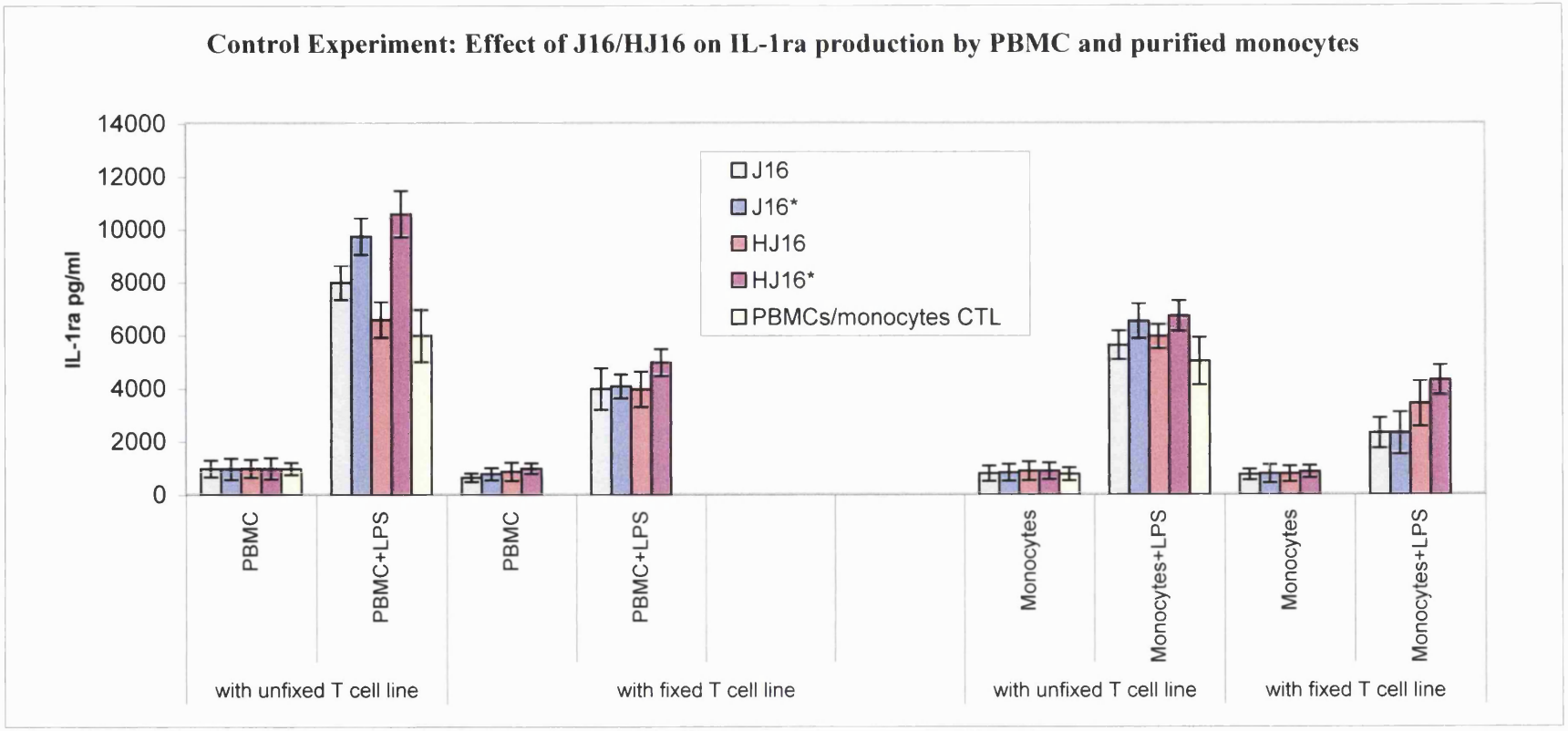


Figure (3.9b) ELISA results of PBMC and monocytes, illustrates a similar pattern of IL-1ra produced by PBMC and purified monocytes. The result is the mean of three experiments (+/- SEM), (*= Activated cells with PDBu and ionomycin).

3.1.5.3 IL-1β and IL-1ra production by THP-1 cells

J16, HJ16, HJ16 clones (activated/unactivated) (fixed or unfixed) were co-cultured with differentiated THP-1 cells (with/without LPS) for 48 hours at 37°C and 5% CO_2.

Like PBMCs, LPS induced the production of both IL-1β and IL-1ra production in control THP-1 cells. In the absence of LPS insignificant amount of both IL-1β and IL-1ra was detected. As expected, the amounts of IL-1 β and IL-1ra release by THP-1 cells were much less than the amount released by fresh PBMCs.

In the presence of LPS, unfixed activated T cell line induced much higher level of IL-1β (Figure 3.10a) compared to fixed (Figure 3.10b) or un-activated cells. Like the pattern observed in PBMCs culture, activated, unfixed HJ16 and HJ16 clones were a better stimulant to IL-1β production by THP-1 cells compared to J16 cells ($p<0.05$). No significant differences among the clones were observed in the stimulation of IL-1β and IL-1ra production by PBMCs or THP-1 cells.

A reverse pattern for IL-1ra was obtained, in the presence of LPS, fixed and activated T cell line (Figure 3.10 d) induced more IL-1ra compare to unfixed cells. Unfixed T cell line provided limited stimulation to IL-1ra production by THP-1 cells. Fixed, activated HJ16 cells and HJ16 clones induced slightly higher levels of IL-1ra than fixed and activated J16 cells.

In the presence of LPS, the levels of IL-1ra was significantly higher than IL-1β, in a ratio of approximately 15:1

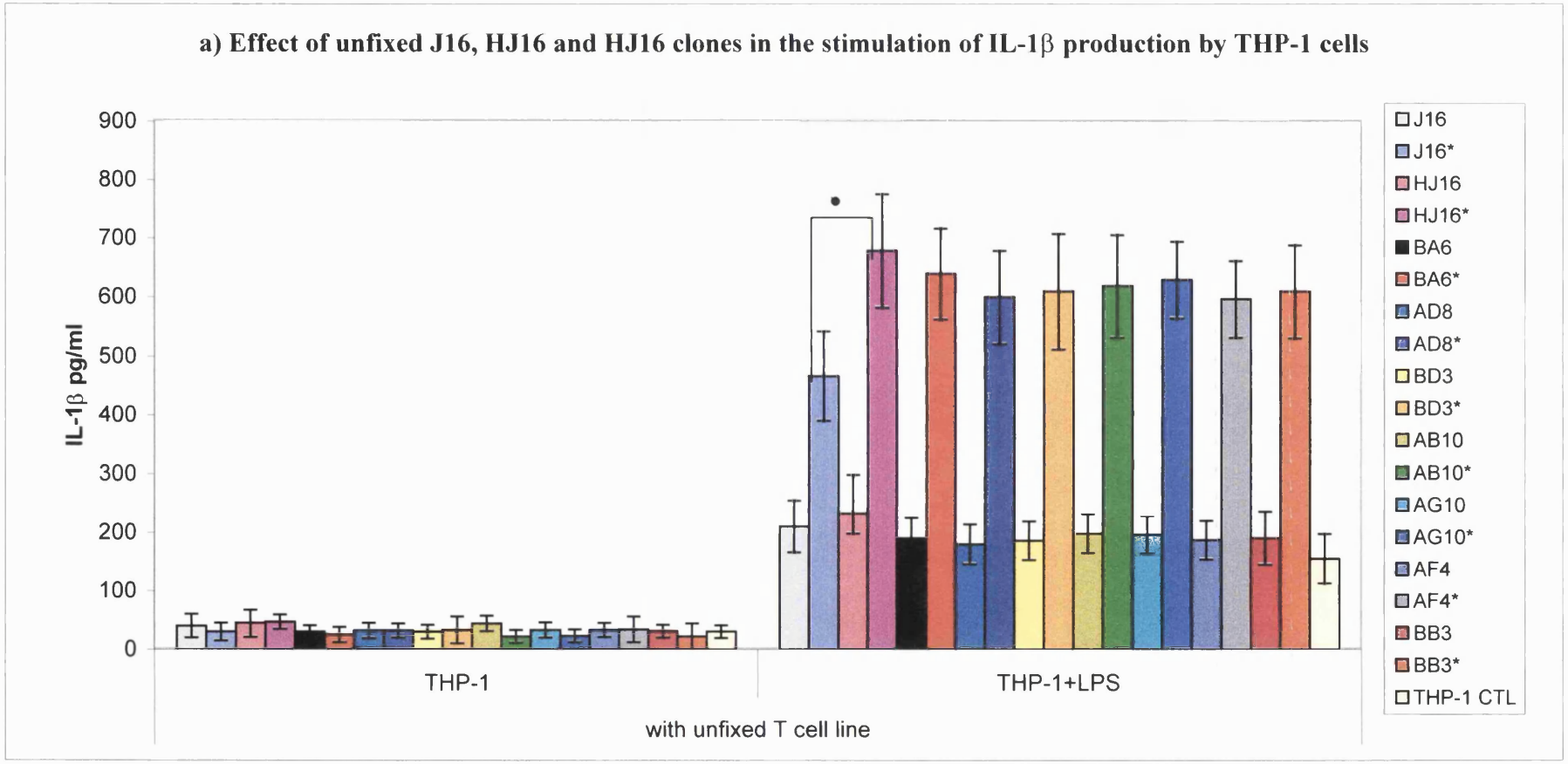


Figure 3.10a: ELISA results of IL-1β from various co-cultures of unfixed J16, HJ16 and HJ16 clones co-cultured with differentiated THP-1 cells. Both HJ16 and the clones of HJ16 cells stimulated more IL-1β production by THP-1 cells in the presence of LPS compared to J16 cells. The data shown is the mean of seven experiments (+/-SEM), (*= Activated cells with PDBu and Ionomycin, • indicates p value <0.05).

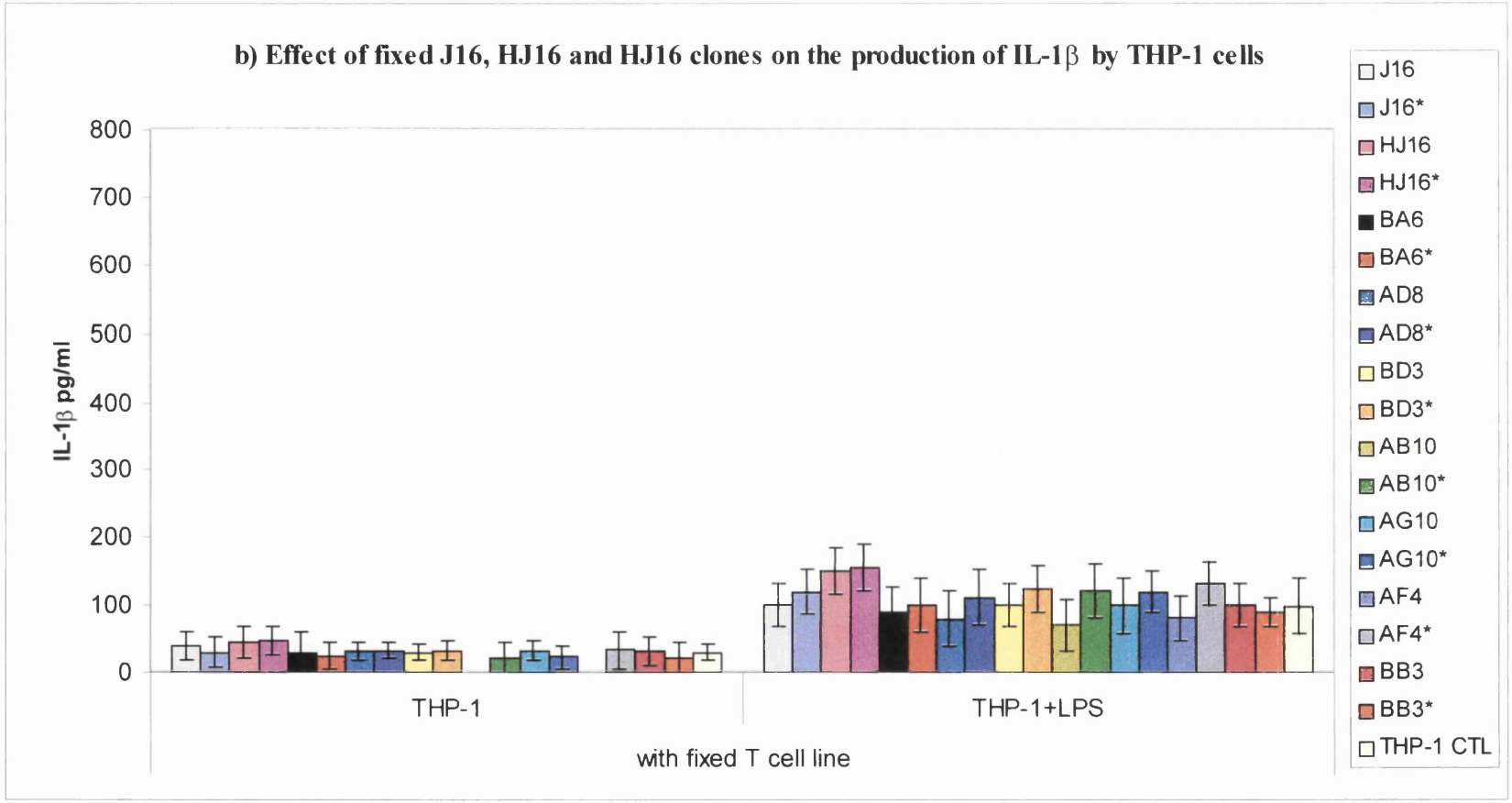


Figure 3.10b: ELISA results of IL-1β from various co-cultures of fixed J16, HJ16 and HJ16 clones co-cultured with differentiated THP-1 cells. No significant stimulation of IL-1β production by THP-1 cells obtained with fixed T cell line. Data shown are the mean of seven experiments (+/-SEM), (*= Activated cells with PDBu and Ionomycin).

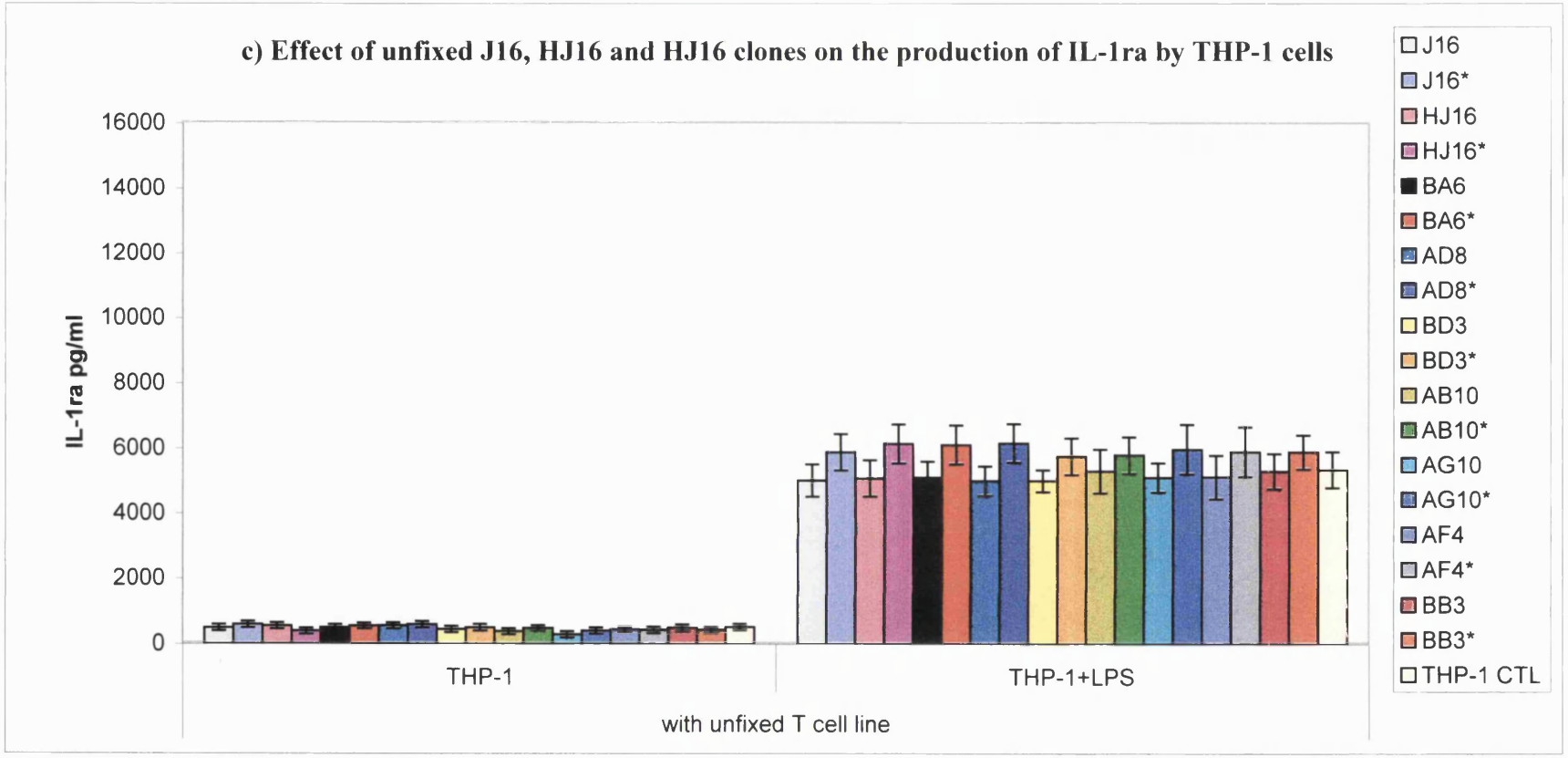


Figure 3.10c: ELISA result of IL-1ra from various co-cultures of unfixed J16, HJ16 cells and HJ16 clones co- cultured with differentiated THP-1 cells. A slight stimulation of IL-1ra production by THP-1 cells was seen with unfixed T cells, activated HJ16 stimulated higher levels of IL-1ra compared to J16 cells. Data shown are the mean of seven experiments (+/- SEM), (*= Activated cells with PDBu and ionomycin).

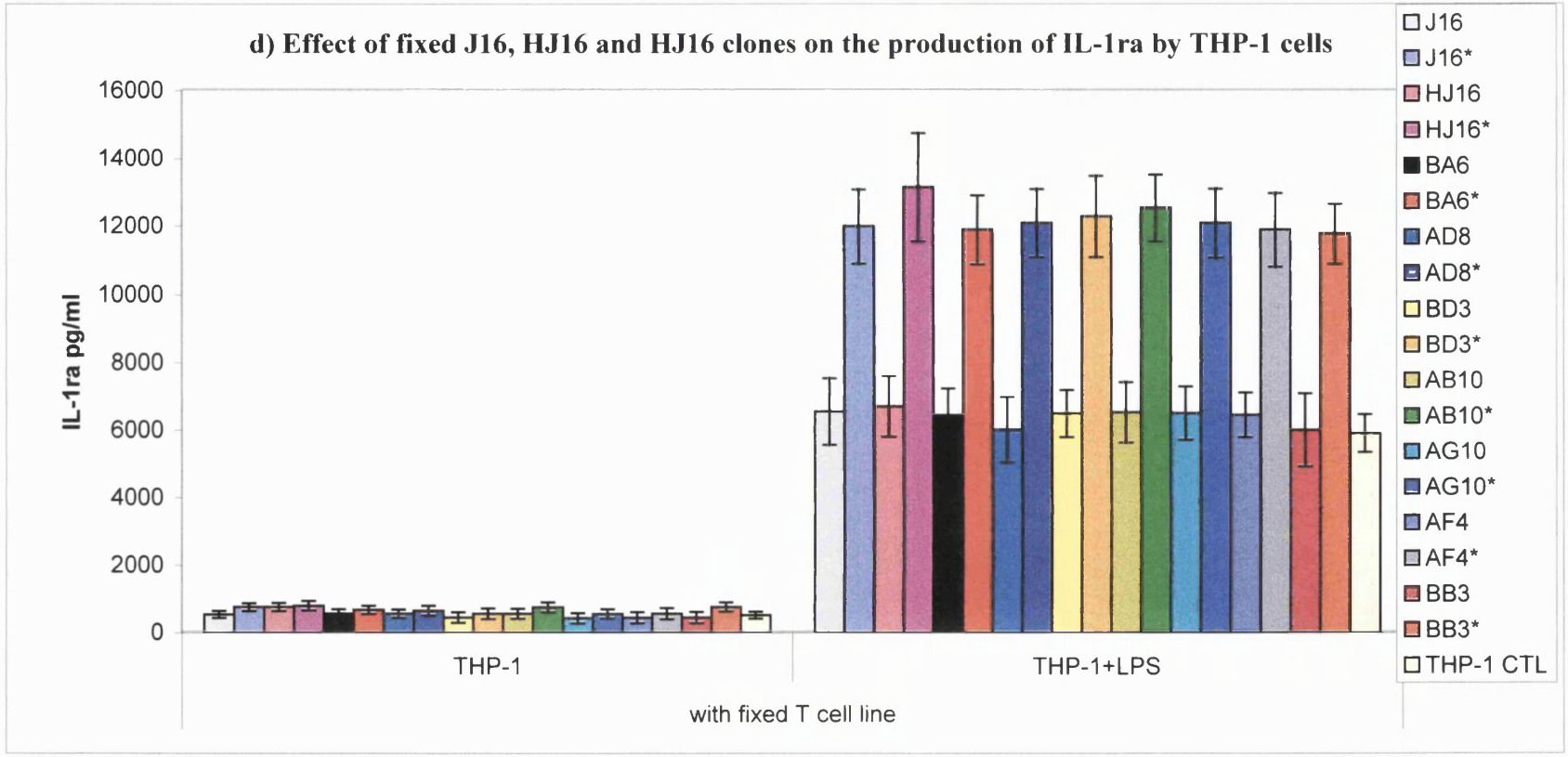


Figure 3.10d: ELISA result of IL-1ra from various co-cultures of fixed J16, HJ16 and HJ16 clones co- cultured with differentiated THP-1 cells. Activated fixed T cells stimulated significantly higher level of IL-1ra compared to unfixed cells. Fixed HJ16 cells and the clones were more active than J16 cells in stimulation of IL-1ra production. Data shown are the mean of seven experiments (+/-SEM), (*= Activated cells with PDBu and ionomycin).

3.2 Effect of various stimulants on J16 and HJ16 cells and the production of IL-1β by THP-1 cells

Several T cells activation pathways were investigated in this section. J16 and HJ16 cells were washed once in PBS, resuspended in RPMI at 1x 10^6 cells/ml and activated with ionomycin, PDBu, ionomycin/PDBu, anti-CD3, anti-CD28, and both anti-CD3/CD28 for 24hours. Cells were then washed three times in PBS and resuspended in RPMI at $5x10^6$ cells/ml and co-cultured with differentiated THP-1 cells in the presence of LPS. Results are presented in Figure 3.11a,b.

Ionomycin, PDBu alone and 1μg/ml CD3 showed a limited effect on the stimulation of THP-1 cells to produce IL-1β or IL-1ra compared to the combination of ionomycin and PDBu, anti-CD3 (10μg/ml), combination of CD3/CD28 (10μg/ml), and PHA-L (10 μg/ml), which all significantly enhanced the production of IL-1β and insignificantly IL-1ra production by THP-1 cells. HJ16 cells at all conditions (except ionomycin, PDBu alone and 1μg/ml CD3) provided better stimulation for the production of both IL-1β ($p<0.05$) and IL-1ra (but not at significant levels) than J16 cells.

Activation of T cell line (J16 and HJ6 cells) with combination of CD3 and CD28 antibodies or ionomycin and PDBu resulted in significantly higher levels of IL-1β production by THP-1 cells than activation of T cell line with PHA-L, CD3, and CD28 antibody ($p< 0.05$).

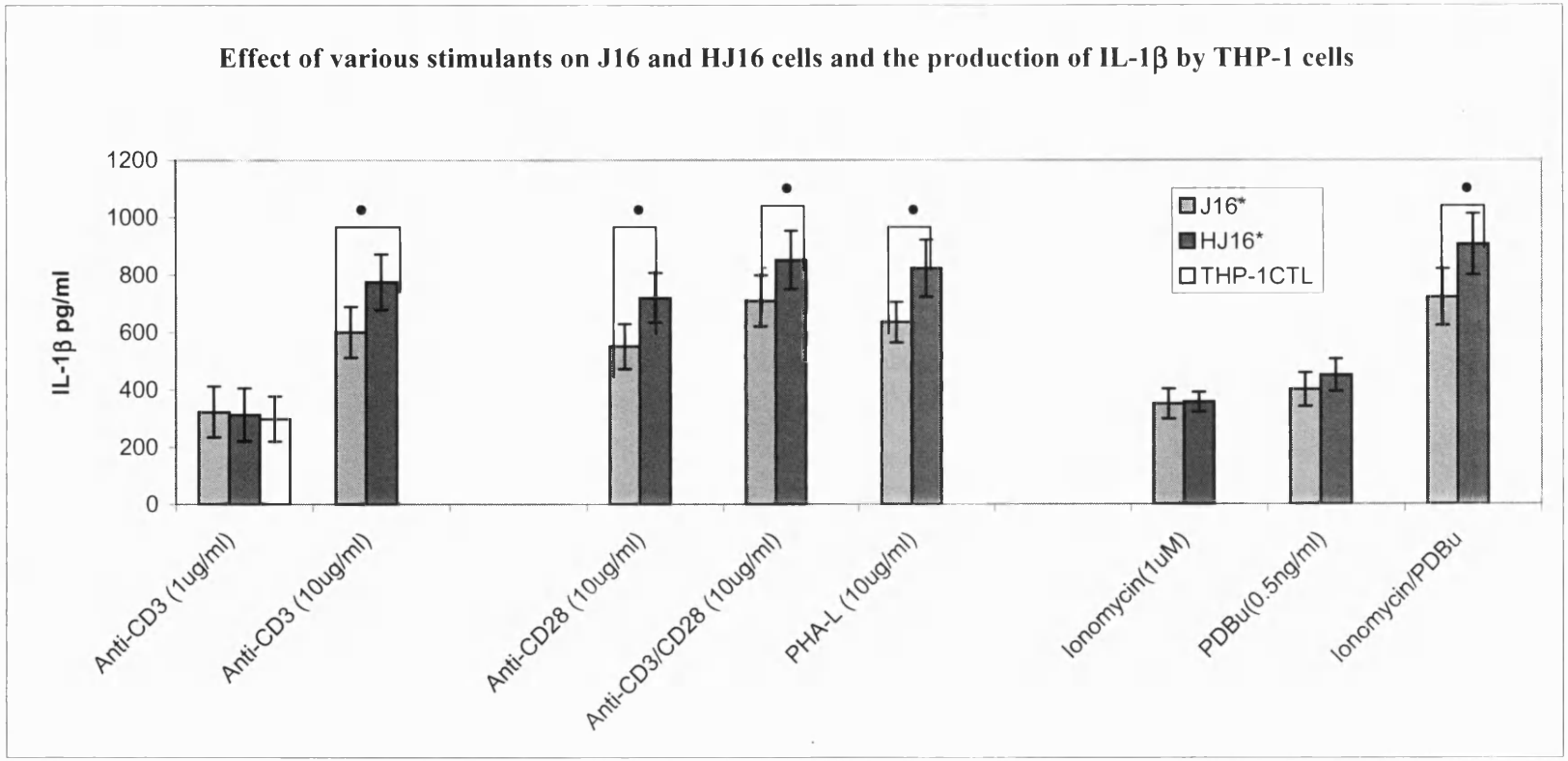


Figure 3.11a: Effect of various stimulants on J16 and HJ16 and the production of IL-1β, activated (*) HJ16 cells with ionomycin/PDBu, anti-CD3, anti-CD28, combination of anti-CD3 and CD28 (10μg/ml), or PHA-L significantly stimulated IL-1β production by THP-1 cells compared to activated J16 cells ($p<0.05$, indicated as •). Ionomycin, PDBu alone or 1μg/ml anti-CD3 had no or limited effect on both J16 or HJ16 cells. Results represented as the mean value of four experiments.

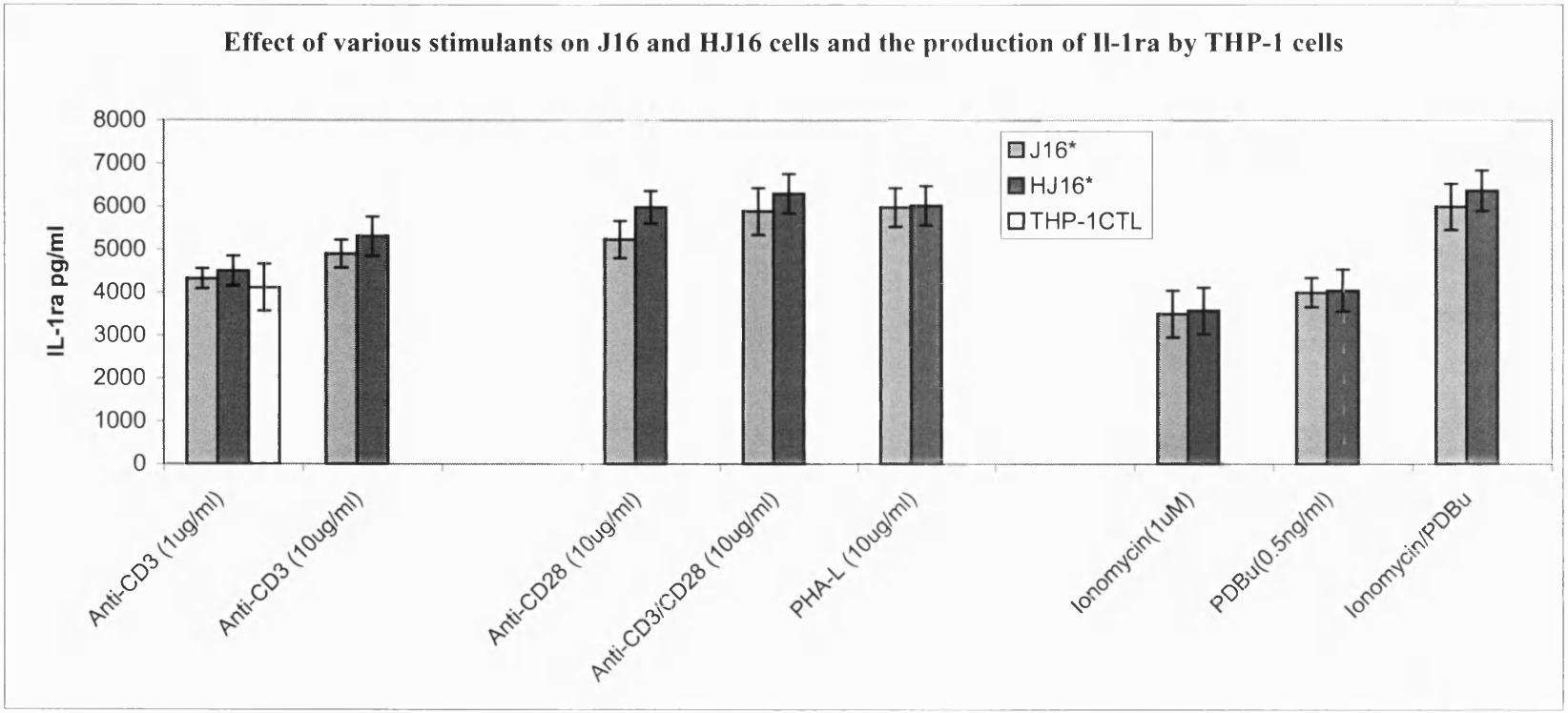


Figure 3.11b: Effect of various stimuli on J16 and HJ16 cells, and the production of IL-1ra by THP-1 cells, unfixed and activated (*) HJ16 cells with various stimuli slightly stimulated IL-1β production by THP-1 cells compared to activated J16 cells or the control cells. Results represented as the mean value of four experiments.

3.3 Effect of supernatants of J16 and HJ16 cells on the production of IL-1β by THP-1 cells

In order to investigate the involvement of any soluble factor (s) produced by J16 and HJ16 cells during the stimulation of IL-1β production by THP-1 cells, supernatants from activated J16 or HJ16 cells were incubated with THP-1 cells. Cells were activated with ionomycin/PDBu, anti-CD3 or PHA-L overnight, cells incubated with ionomycin/PDBu were washed three times and incubated further 24hours, cells activated with anti-CD3 or PHA-L were centrifuged and the supernatants were harvested. Supernatants of activated J16 and HJ16 cells were incubated with THP-1 cells in the presence of LPS.

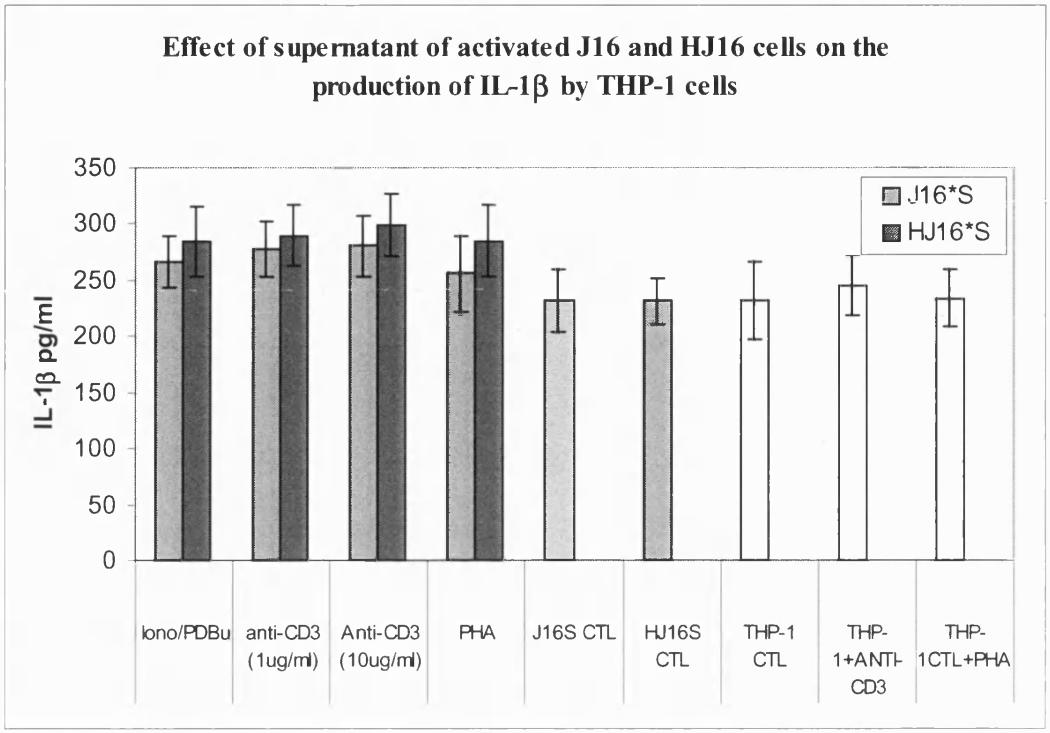
As shown in Figure 3.12, activated J16 and HJ16 supernatant enhanced the production of IL-1β by THP-1 cells. Activated HJ16 supernatant (HJ16*S) was more active, in term of stimulation of IL-1β production than activated J16 supernatant (J16*S). This was manifested by a moderate increase of IL-1β production by THP-1 cells, but statistically not significant (P=0.0625).

J16*S, HJ16*S from 1 μg/ml anti-CD3 activation, had no or limited effect on IL-1β production, and no stimulation of IL-1β was observed with un-activated J16/HJ16 supernatant. Anti-CD3 or PHA-L also had no effect on THP-1 control cells, Figure 3.12a.

Supernatant results indicated a similar pattern to cells in previous experiments, suggesting that soluble factors are involved in co-culture. However, from this result, it seems that the soluble factors are not only factors that mediate IL-1β production,

hence that, the levels of IL-1β produced by THP-1 cells under the stimulation of the supernatants of J16 or HJ16 cells are much less than that produced by THP-1 cells co-cultured with J16 or HJ16 cells.

Supernatants of un-activated or activated J16 or HJ16 cells failed to provide stimulation for IL-1ra production by THP-1 cells. There was no significant difference between the control of THP-1 cells and that with supernatants, as shown in Figure 3.12b. This indicates that cell-cell contact is essential mechanism that mediates the production of IL-1ra by THP-1cells.



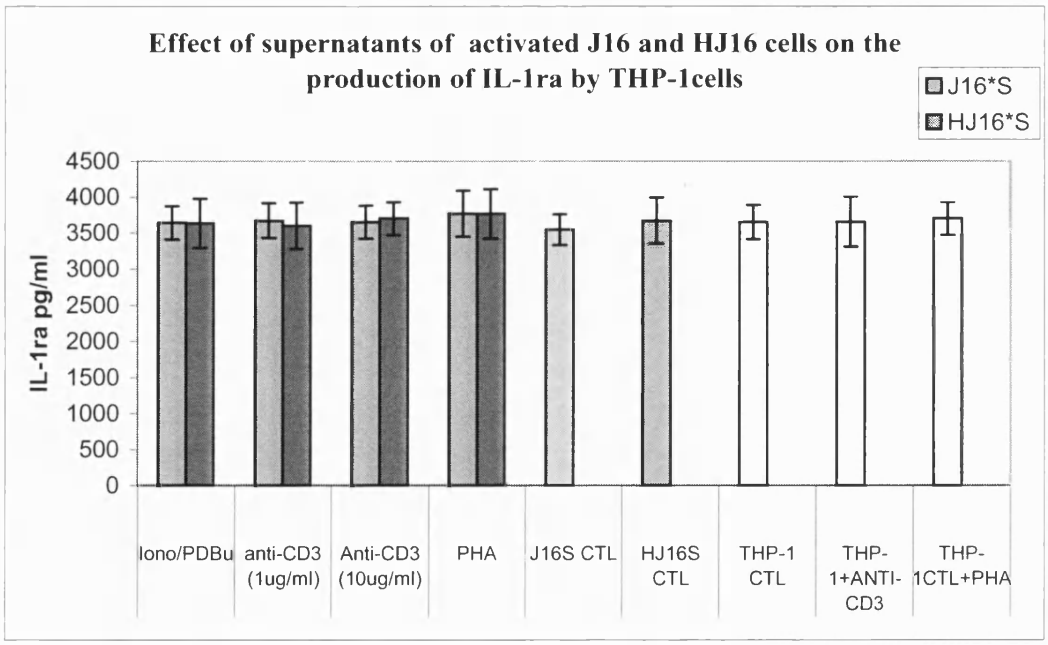


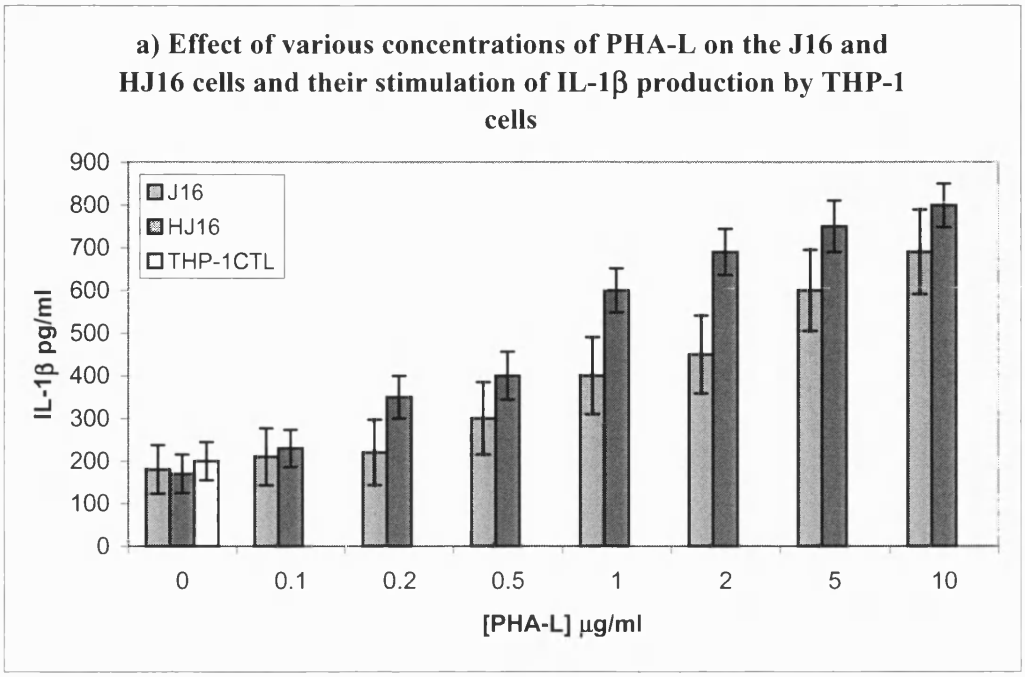
Figure 3.12a: Effect of supernatant of J16/HJ16 cells activated with ionomycin (1μM/ml)/PDBu (0.5ng/ml), anti-CD3 (1μg/ml & 10μg/ml) on the production of (a) IL-1β and (b) on IL-1ra by THP-1 cells. Supernatants of both activated J16 and HJ16 cells stimulated IL-1β production by THP-1 cells, but not at significant level (p=0.0625), and failed to stimulate IL-1ra production. Values are the mean of three experiments (+/-SEM), (*=activated cells).

3.4 Study of sensitivity of J16 and HJ16 cells to PHA-L in co-culture

J16 and HJ16 cells were activated with various concentrations of PHA-L (0.1-10μg/ml) for 10 hours, and co-cultured with THP-1 cells for 48 hours in the presence of LPS. Supernatants of the co-cultured were harvested and IL-1β and IL-1ra levels were measured by ELISA.

Activation of J16 and HJ16 cells with various concentrations of PHA-L augmented the release of IL-1β and IL-1ra by THP-1 cells in a dose-dependent manner. Insignificant stimulation of IL-1β (Figure 3.13a) production by THP-1 cells was seen with HJ16 cells activated with 0.1 μg/ml, and no stimulation was observed with J16 cells activated with the same concentration or with 0.2μg/ml. However, a significant level ($p<0.05$) of IL-1β was obtained when HJ16 cells stimulated with 0.2 –10 μg/ml PHA-L compared to J16 cells. J16 cells responded to concentration of 0.5-10 μg/ml PHA-L (or above), but their stimulation to IL-1β production by THP-1 cells was significantly less than the stimulation observed with HJ16 cells at all concentration of PHA-L.

HJ16 cells activated with 0.1-10 μg/ml also induced higher levels of IL-1ra by THP-1 cells than activated J16 cells, but this is not statistically significant (Figure 3.13b).



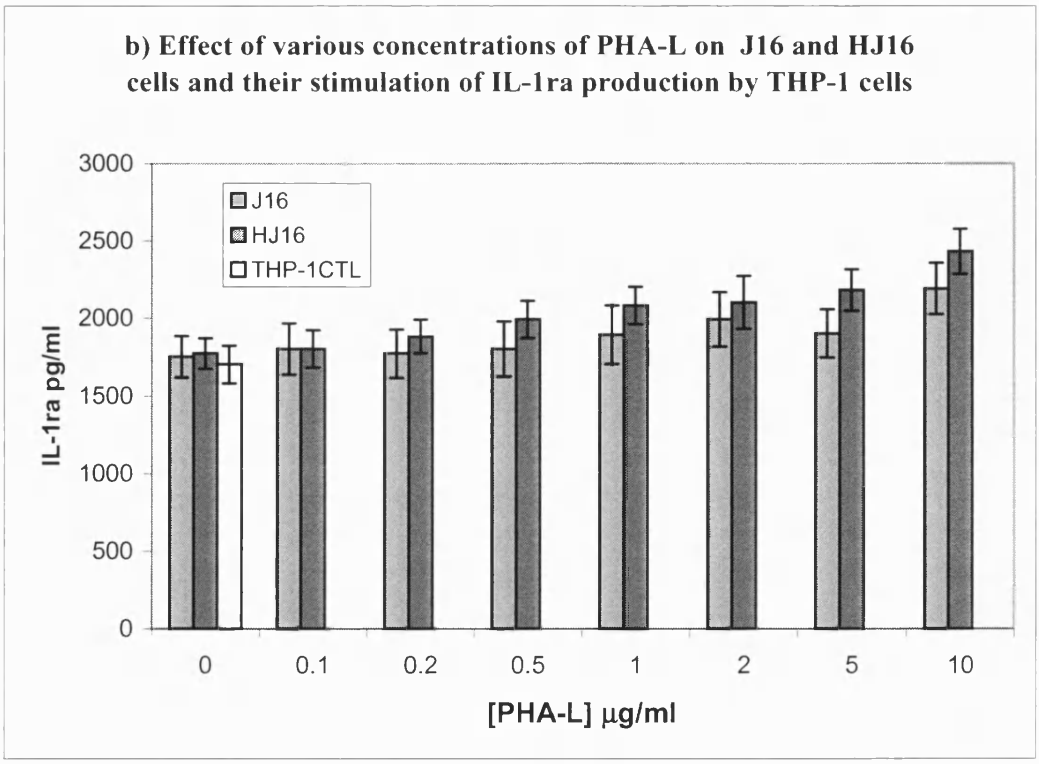


Figure 3.13: Effect of various concentrations of PHA-L on the production of (a) IL-1β and (b) IL-1ra by THP-1 cells. HJ16 cells were more sensitive to low concentrations of PHA-L, manifested in better stimulation of IL-1β ($p<0.05$) and IL-1ra (not statistically significant) production by THP-1 cells. The data presented is the mean of three experiments +/-SEM.

3.5 Comparison between J16 and UV-A resistant-J16 (UVAR-J16), in co-culture with THP-1 cells

It has been documented that UV-A irradiation decreased the ability of epidermal cells to present antigen to T cells directly. This effect was accompanied by suppression of the expression of accessory molecules (ICAM-1, B7.1, B7.2). Also, it has been noted that, UVA decreased the proliferation of lymph node cells, (Iwai et al; 1999).

In these experiments, UV-A resistant Jurkat cells (UV-A-R-J16) were used in comparison with the normal Jurkat cell line, to assess the effect of UV-A on these cells and their stimulation to IL-1β and IL-1ra by THP-1 cells.

Cells were activated with ionomycin/PDBu overnight, washed and co-cultured with THP-1 cells. In contrast to HJ16 cells, no significant difference was seen between J16 and UV-A-R J16 cells in stimulation of IL-1β and IL-1ra production

All fixed cells were better stimulants of IL-1ra production than unfixed cells. However no significant difference between fixed J16 and fixed UVAR-J16 was seen in the stimulation of IL-1ra production by THP-1 cells (Figure 3.14).

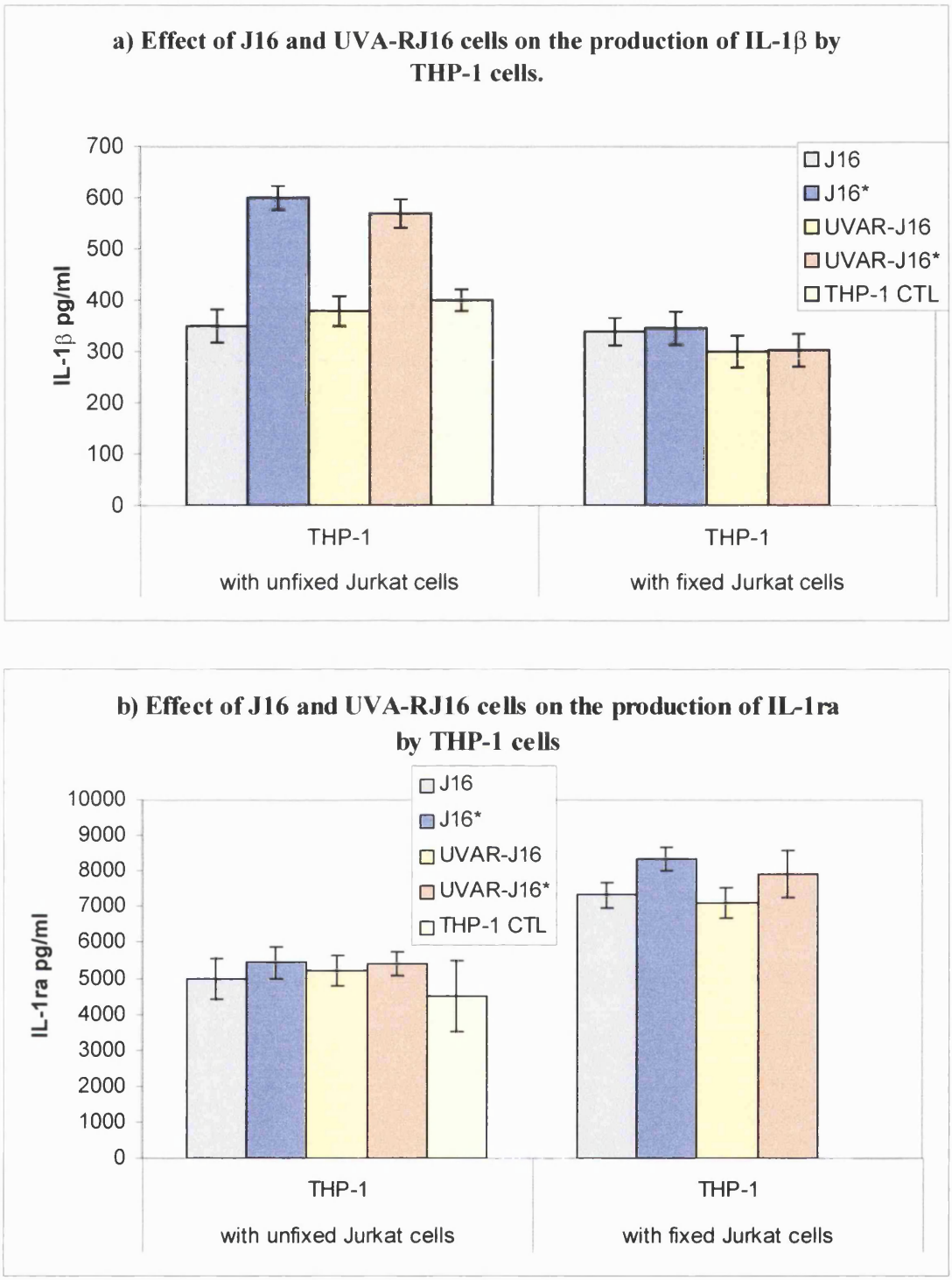


Figure 3.14: Comparison between the effect J16 and UVAR-J16 cells on the production of (a) IL-1β and (b) IL-1ra by THP-1 cells. No significant difference between UVAR-J16 and J16 in stimulation of both IL-1β /IL-1ra, (*=Activated cells with ionomycin/PDBu). The data is the mean of three experiments, +/- SEM.

3.6 Effect of Hypoxia (1% and 2% oxygen) on the production of IL-1β and IL-1ra by PBMCs

Hypoxia is a common modification of the extracellular environment that can affect cellular function. Hypoxia induces the expression of certain cytokines such as IL-1β and IL-1ra (Naldini, et al; 2001). The effect of hypoxia (1% and 2% O_2) on the production of IL-1β and IL-1ra by PBMCs and their stimulation was investigated. In order to asses the effect of hypoxia on T cells and their stimulation to IL-1β and IL-1ra production by PBMCs, J16 and HJ16 cells were incubated with PBMCs in low oxygen, 1% or 2% O2, 5% CO_2, and normal oxygen, (20 % O_2, 5% CO_2). The production of cytokines was measured in the harvested supernatants, and the results were expressed as the mean value of three experiments.

A significant reduction in IL-1β and IL-1ra production was observed in cells incubated under 1% O_2 (hypoxia) compare to 20% O_2 (normoxia) ($p<0.01$). However, J16 and HJ16 cells provided PBMCs with slight stimulation with respect to controls, but no significant difference between J16 or HJ16 cells stimulation at 1% O2, Figure 3.15a,b. In contrast, 2% O_2 significantly ($p<0.01$) enhanced IL-1β and IL-1ra production by PBMCs in the presence of LPS, and due to substantial increase in IL-1β and IL-1ra in control cells (PBMCs), it was difficult to determine whether J16 or HJ16 cells provided stimulation to PBMCs to produce IL-1β and IL-1ra, Figure 3.15a,b. A slight increase in both IL-1β and IL-1ra production by PBMCs in the absence of LPS was generated by hypoxia (2% O2).

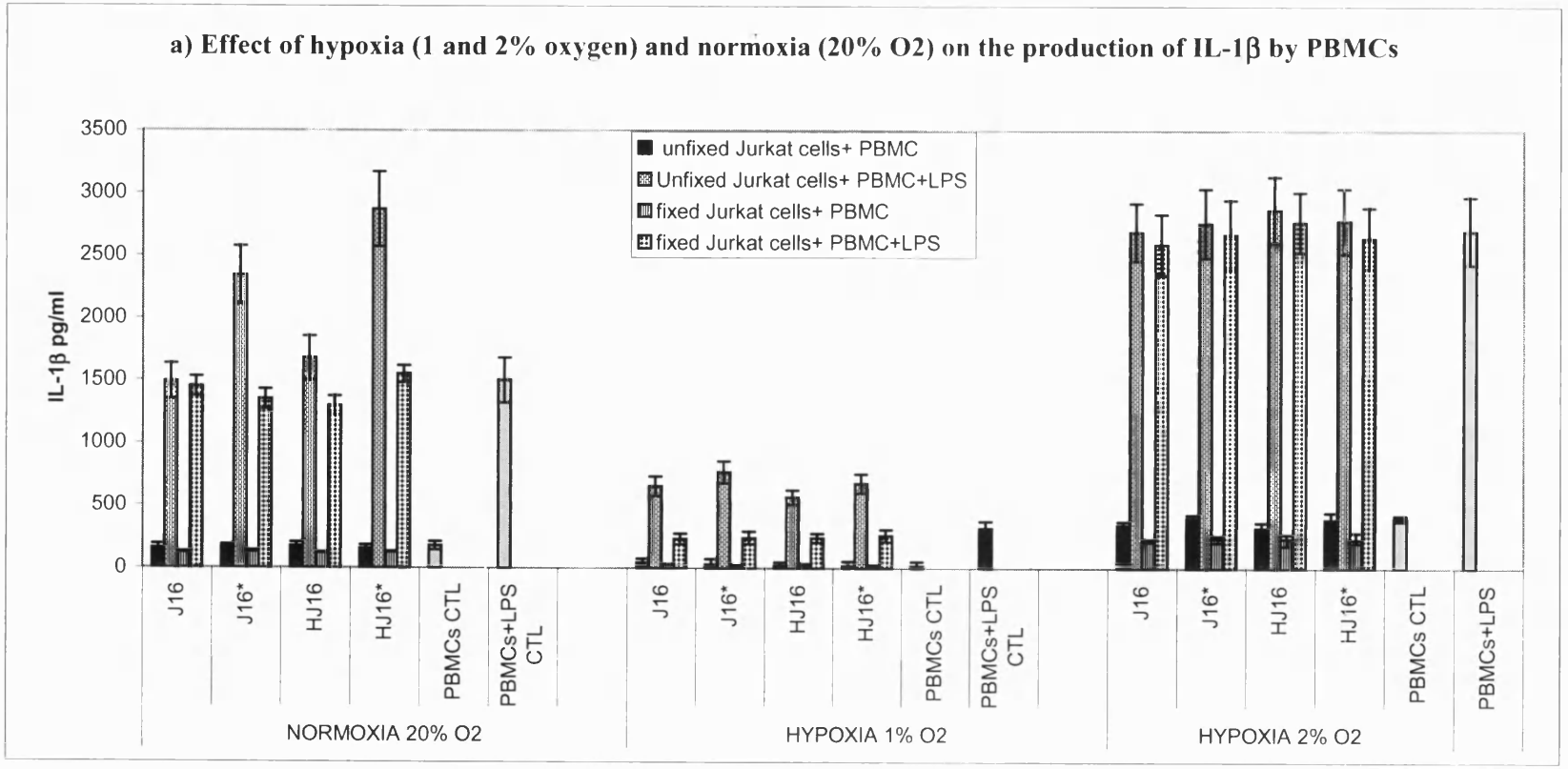


Figure 3.15a: Effect of 1 & 2 % O_2 in the production of IL-1β by PBMCs. A significant increase in IL-1β production was observed with 2% O_2. The results expressed as the mean value of three experiments (+/-SEM).

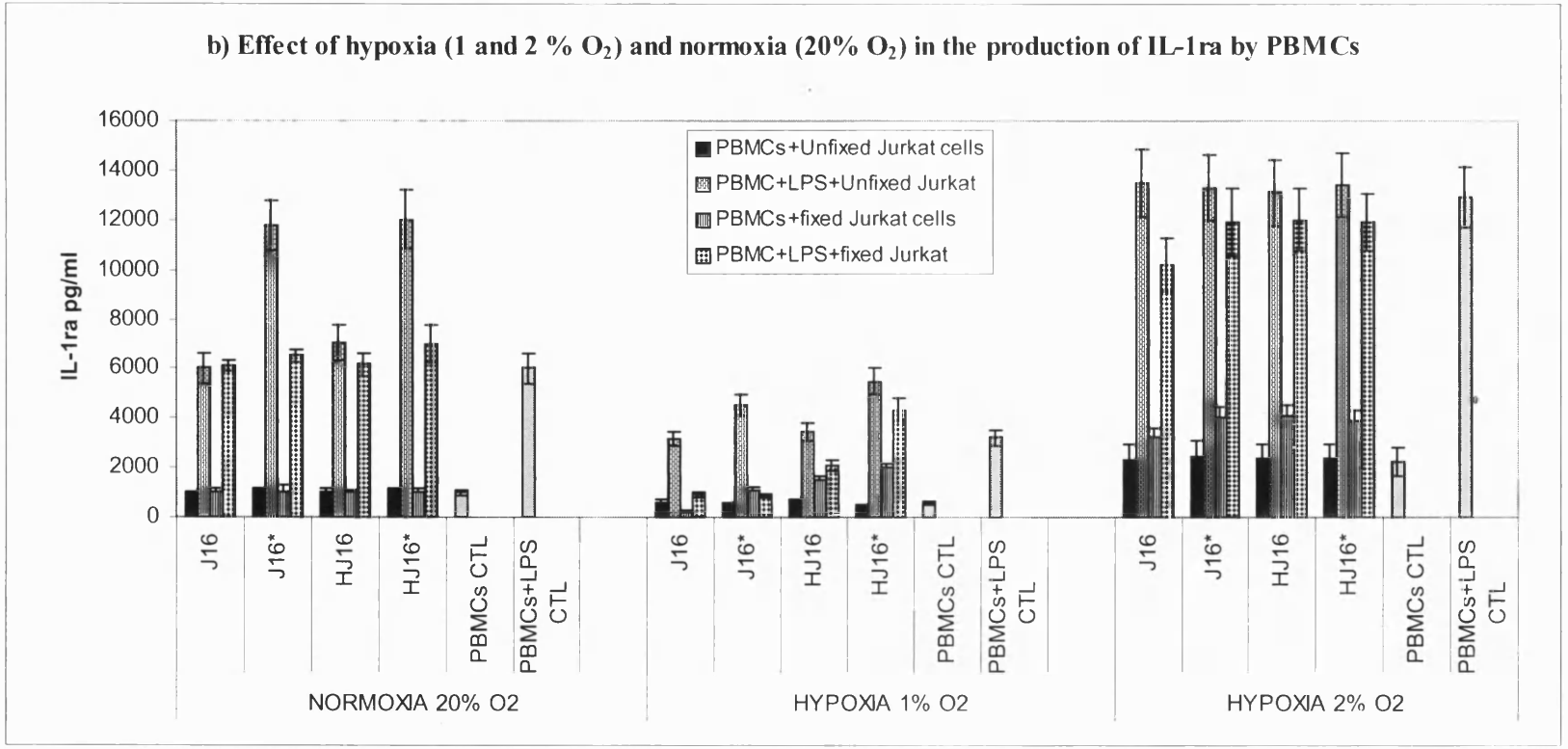


Figure 3.15b: Effect of 1 & 2 % O_2 in the production of IL-1ra by PBMCs. A significant increase in IL-1ra production was observed with 2% O_2. The results expressed as the mean value of three experiments (+/-SEM).

CHAPTER 4

RESULTS

MANIPULATION OF THE INTRACELLULAR ANTIOXIDANT, GLUTATHIONE, IN J16 AND HJ16 CELLS

Chapter 4 Results

4.1 Manipulation of the intracellular antioxidant, glutathione (GSH), in J16 and HJ16 cells

Glutathione (GSH) is the main intracellular defence against oxidative stress, and regulates the redox potential. Modulation of intracellular GSH levels has previously been shown to influence a variety of cell functions. For example, L-buthionine (DL)-sulfoximine (BSO) treatment to decrease GSH levels augmented TNF-α and IL-6 production by epithelial cells (Haddad et al; 2001 & 2002). Moreover, hyporesponsiveness of synovial T cells in rheumatoid arthritis was associated with decreased intracellular levels of GSH and increased levels of thioredoxin (another major cellular antioxidant) (Maurice et al, 1998).

Here we are investigating the effect of decreasing or increasing of GSH levels in J16 and HJ16 cells and their ability to stimulate IL-1β and IL-1ra production by THP-1 cells.

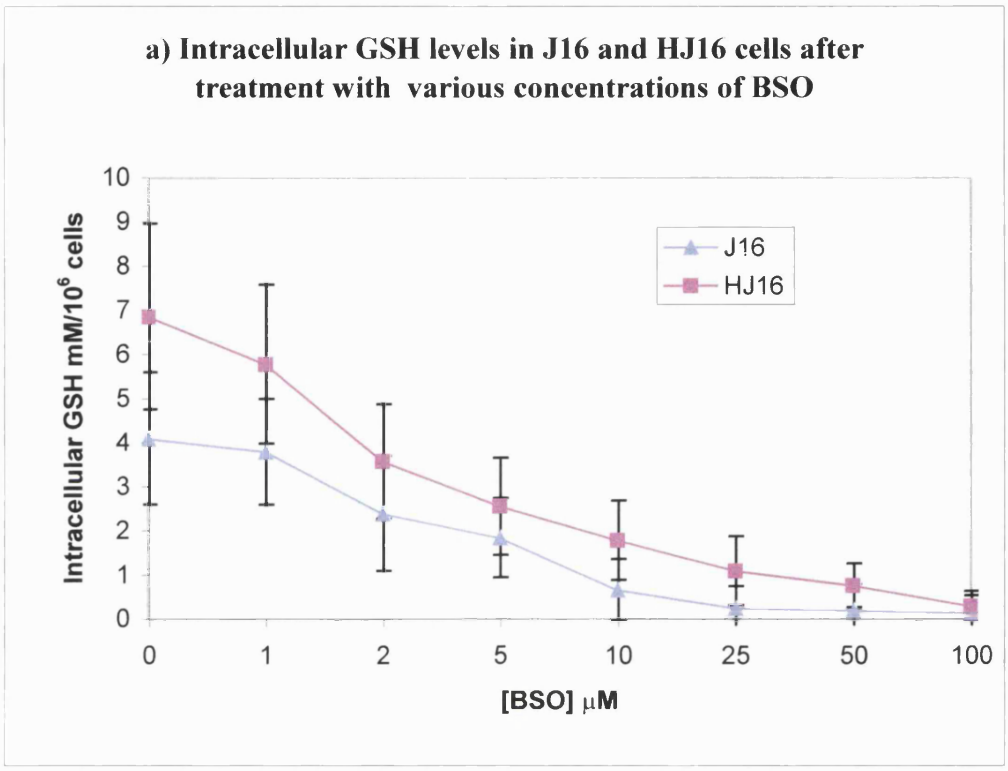
BSO, diethyl maleate (DEM) and diamide were used to decrease GSH, and NAC to increase GSH in J16 and HJ16 cells. Experiments using trypan blue exclusion tests were always performed in order to exclude any toxicity of these drugs.

4.1.1 Measurement of glutathione (GSH) on J16 and HJ16 cells

In order to manipulate intracellular GSH levels, J16 and HJ16 cells were incubated with BSO to deplete GSH, and NAC to increase GSH. Cells were washed once in PBS, resuspended in RPMI at 0.5 x 10^6cells/ml, various concentrations of BSO or

NAC were added, cells were incubated overnight, and intracellular GSH levels were measured.

Interestingly, the HJ16 cell line showed significantly higher GSH content than J16 cells ($p<0.01$). The mean value of three experiments of GSH levels was 6.7 μM for HJ16 cells and 4.2 μM for J16 cells. Addition of 2-100 μM BSO significantly decreased GSH levels in both cell lines in a dose-dependent manner. A slight decrease was seen with both cell types treated with 1μM BSO. As expected, NAC treatment (2.5, 5 and 10 mM) significantly increased GSH levels in both cell lines (Figure 4.1). However, GSH concentrations in HJ16 cells remained higher than J16 cells at all treatments.



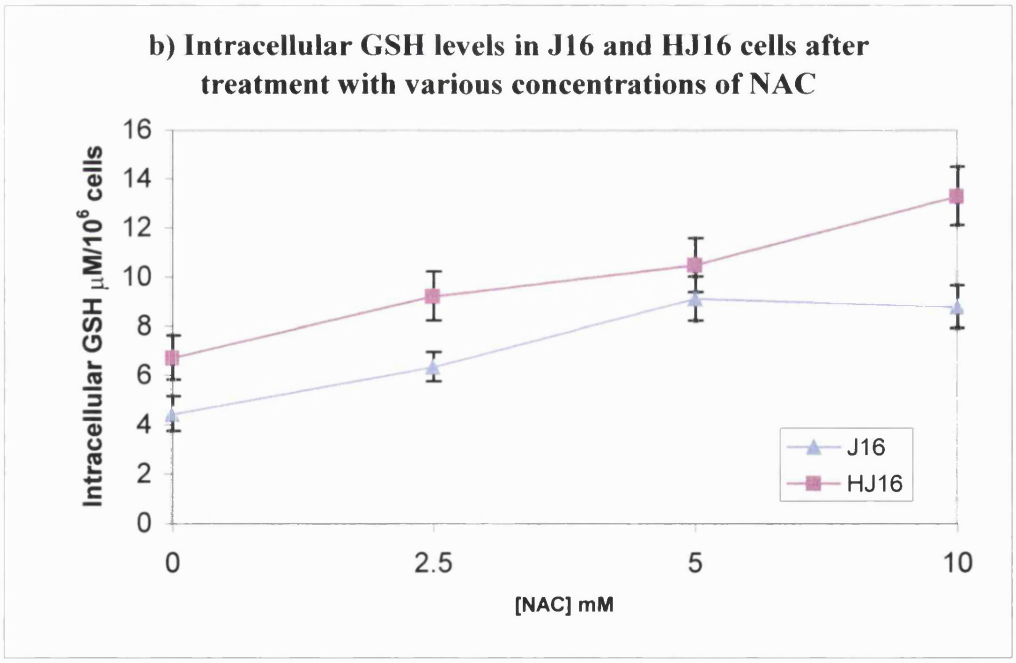


Figure 4.1 Intracellular GSH content of J16/HJ16 cells. Cells were treated with various concentration of a) BSO or (b) NAC for 24 hours and presented for biochemical determination of intracellular GSH. Results shown are the mean of three separate experiments (+/-SEM).

4.1.2 Investigation into the effect of depletion of GSH in J16 and HJ16 cells and their stimulation of IL-1β/ IL-1ra production by THP-1 cells

GSH levels were decreased in J16 and HJ16 cells by incubating them with various concentrations of BSO, DEM and diamide. The cells were then washed three times and activated with ionomycin/PDBu for 24 hours. They were co-cultured with differentiated THP-1 cells in presence of LPS for 48hours, supernatants were harvested, and IL-1β and IL-1ra production was measured by ELISA. The results are presented as the mean value of several experiments, each performed in duplicate.

Interestingly, depletion of GSH with BSO (50, 100 and 200 μM) in activated J16 and HJ16 cells significantly enhanced the stimulation of IL-1β and production by THP-1cells ($p<0.05$) compared to untreated cells. A moderate increase in IL-1ra production by THP-1 cells was also seen after BSO treatment of J16 and HJ16 cells, but not statistically significant. No significant difference was observed between BSO-treated J16 and HJ16 cells in the stimulation of IL-1β and IL-1ra production by THP-1 cells (Figure 4.2a,b).

To assess the effect of increased GSH levels, cells were incubated with NAC (2.5, 5 and 10 mM) overnight, washed and activated with ionomycin/PDBu, after that cells were washed and co-cultured with THP-1 cells for 48 hours. IL-1β and IL-1ra levels were measured in the harvested supernatants. Unlike BSO-treatment, NAC treatment of these cells resulted in a slight reduction of IL-1β and IL-1ra production by THP-1 cells. This reduction was better seen with J16 cells than HJ16 cells (Figure 4.3a,b).

Although this reduction is not significant, it reveals that there was no enhancement of stimulation of IL-1β or IL-1ra production by THP-1 cells co-cultured with the NAC-treated T cell line.

In order to confirm that the enhancement of the stimulation of IL-1β and IL-1ra production was due to the effect of depletion of GSH levels, diamide and DEM were used as alternative methods to deplete GSH levels in J16 cells. Both methods of depletion of GSH levels resulted in similar results to those obtained with BSO-treated J16 and HJ16 cells. Depletion of GSH with DEM (1mM and 100 μM, for 2 or 24 hours) and diamide (100μM) resulted in a significant increase in the stimulation of IL-1β and to lesser extent IL-1ra production by THP-1 cells (Figure 4.4a,b).

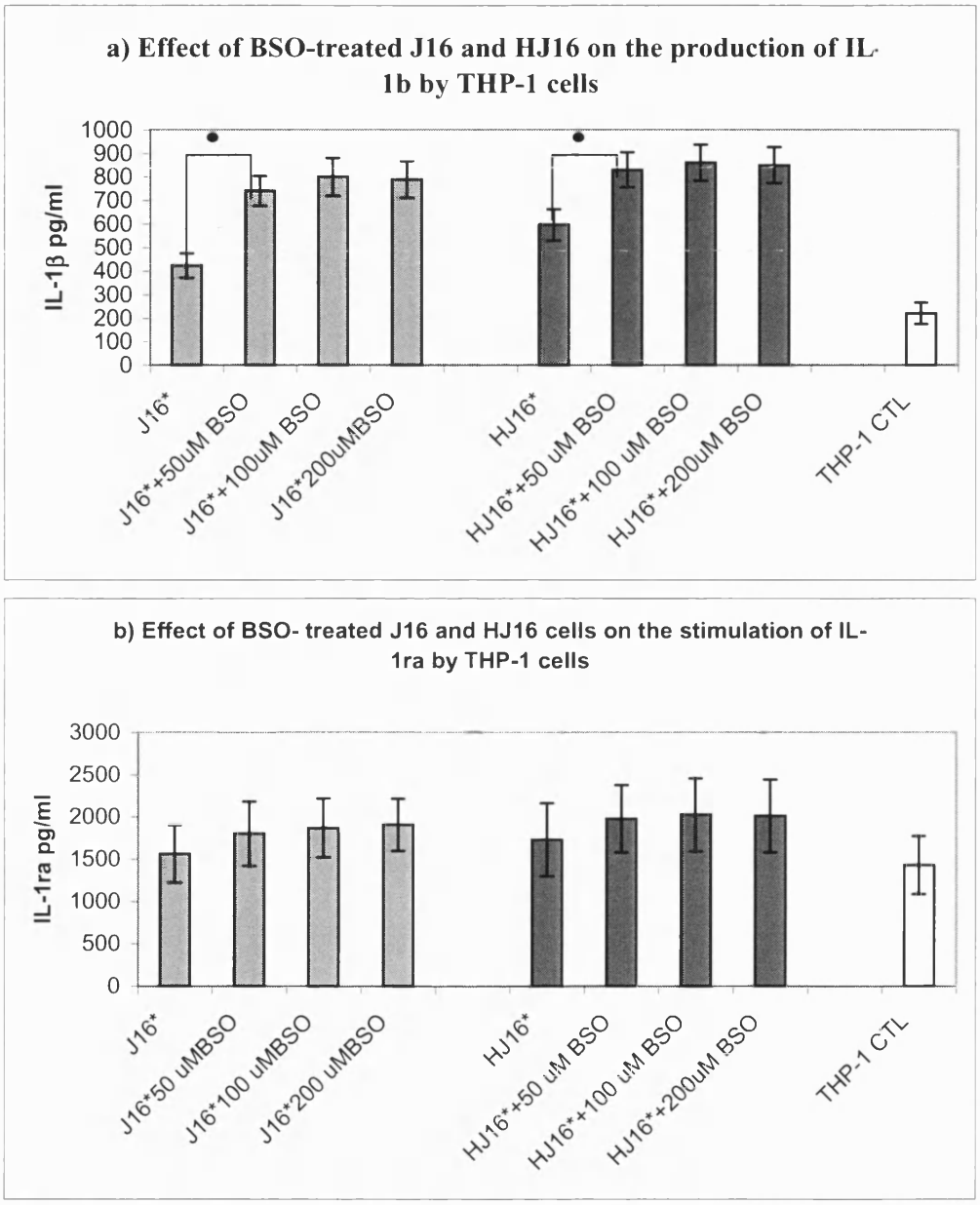
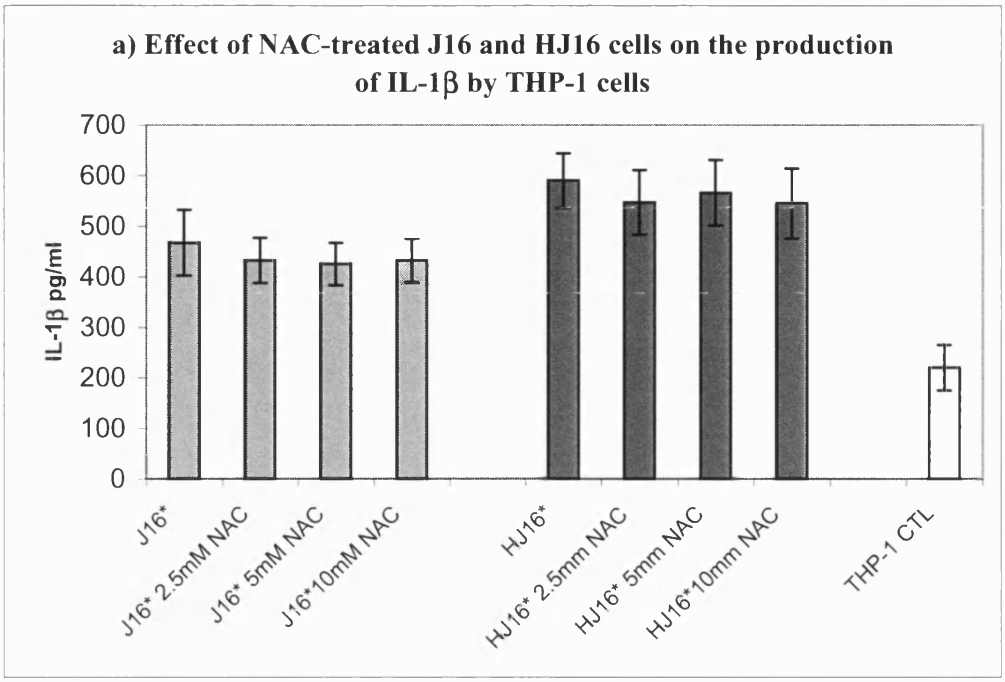
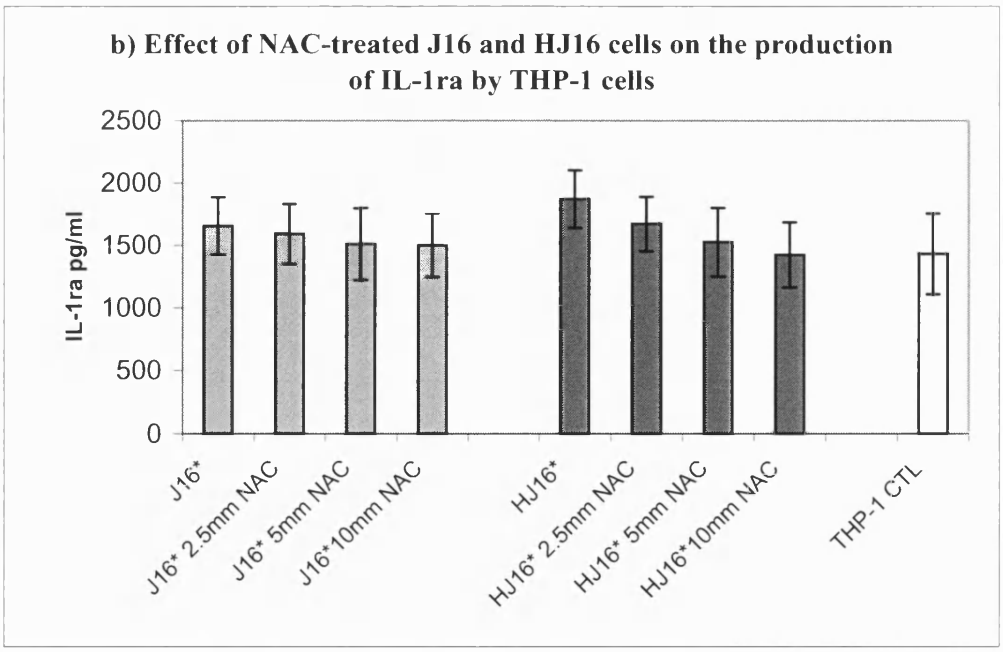
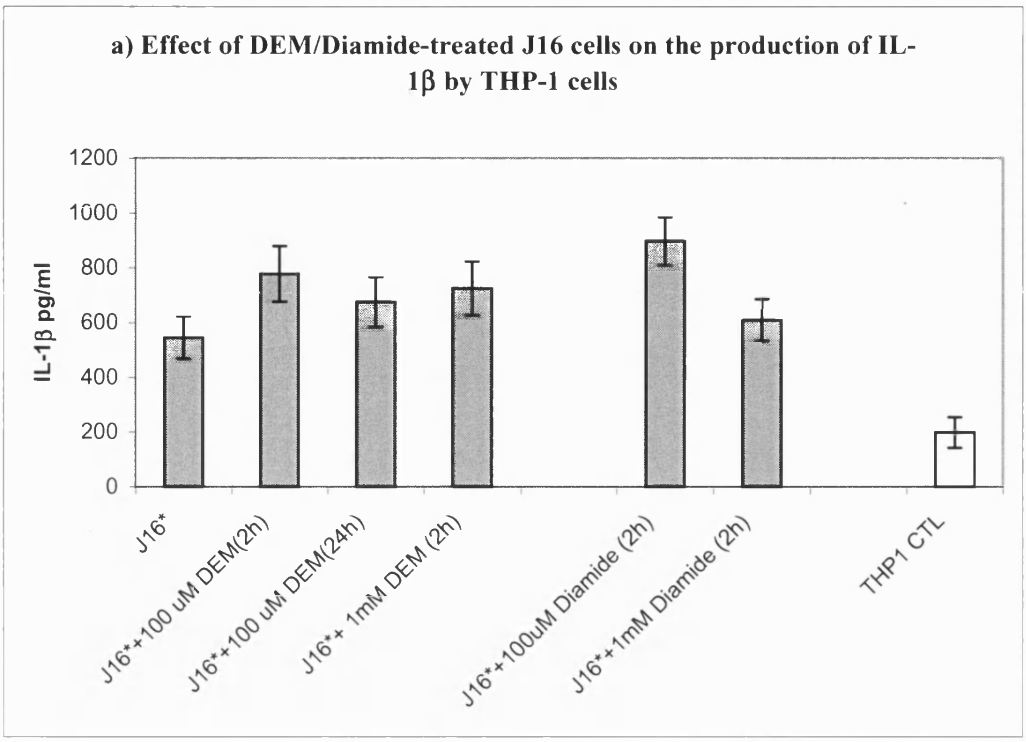


Figure 4.2: Effect of BSO-treated J16 and HJ16 cells on the stimulation of (a) IL-1β and (b) IL-1ra production by THP-1 cells. J16 and HJ16 cells were treated with various concentrations of BSO for 24 hours. BSO treated cells significantly enhanced IL-1β production by THP-1 cells. Low increase in IL-1ra production was obtained. The results presented are the mean value of 5 different experiments (+/-SEM), (*= activated cells with ionomycin/PDBu, • indicates p value< 0.05).





4.3: Effect of NAC-treated J16 and HJ16 cells on the stimulation of (a) IL-1β and (b) IL-1ra production by THP-1 cells. J16/HJ16 cells were treated with various concentrations of NAC for 24 hours. NAC-treated cells resulted in a slight reduction in both (a) IL-1β and (b) IL-1ra production by THP-1 cells. The data presented is the mean of five different experiments, +/- SEM, (*= Activated cells ionomycin/PDBu).



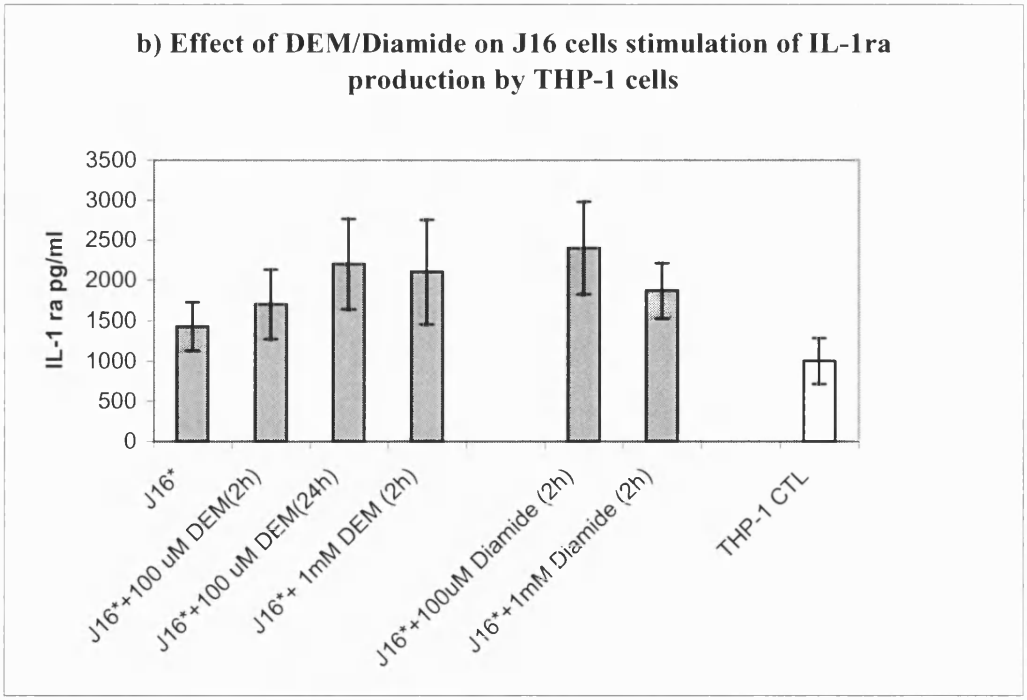


Figure 4.4: Effect of depletion of GSH on J16 cells and their stimulation to (a) IL-1β and (b) IL-1ra: a significant increase in IL-1β and IL-1ra after depletion of GSH with DEM/diamide. The results are the mean value of four experiments, +/- SEM, *= activated cells ionomycin/PDBu.

4.2 Effect of MAP kinase inhibitors on J16 and HJ16 cells and their stimulation of IL-1β and IL-1ra production by THP-1 cells

The availability of chemical compounds that can specifically block one or another signal transduction pathway has greatly contributed to the study of the role of MAP kinases. A newly discovered inhibitor, SP600125, is used to inhibit JNK. It has been shown that SP600125 completely blocks IL-1-induced expression of c-Jun and collagenase mRNA and prevents the accumulation of phosphorylated-Jun in cultured synovial cells (Han et al; 2001). PD98059 can specifically inhibit MEK1/2, two enzymes that phosphorylate and activate ERK1/2. Moreover, P38 can be inhibited by SB203580, addition of this inhibitor blocks the cytokine-induced matrix metalloproteinase release from chondrocytic cells (Vincenti et al; 2001).

Treatment of cells with PDBu leads to activation of protein kinase C (PKC), which is essential in T cell signalling. PKC is also sensitive to oxidants (Gopalakrishna et al; 2000), and can be inhibited by bisindolylmaleimide (BIM) (Toullec et al, 1991).

The effect of these inhibitors on J16 and HJ16 cells before and after treatment with BSO (manipulation of intracellular redox) and their stimulation of IL-1β production by THP-1 cells was investigated. IL-1ra was excluded due to the low level produced by THP-1 cells co-cultured with unfixed Jurkat cells (see chapter 3). J16 and HJ16 cells were incubated with BIM (500nM), PD98059 (20μM), SB203580 (1μM), SP600125 (20μM), and various combination of PD98059, SB203580, and SP600125 for 1 hour prior to activation with ionomycin/PDBu for 24 hours, (concentrations of these compounds were recommended by the suppliers). Cells were then washed in PBS, resuspended at $5x10^6$cells/ml in RPMI and co-cultured with THP-1 cells in the

presence of LPS. Supernatants were analysed for IL-1β and IL-1ra production by ELISA (Figure 4.6).

Interestingly, both activated J16 and HJ16 cells pre-treated with PD98059 inhibitor showed a significant reduction in the stimulation of IL-1β by THP-1 cells ($p<0.05$) compared to untreated cells. However PD98059-treated HJ16 showed slightly higher stimulation of IL-1β than PD98059-treated J16 cells, indicates that HJ16 MAPK ERK1/2 may be up- regulated.

Using SB203580 or SP600125 alone and BIM with J16 and HJ16 cells resulted in a slight decrease in IL-1β production by THP-1 cells. Combination of SB203580 and SP600125 in both cell lines significantly reduced IL-1β production by THP-1 cells ($p<0.05$). HJ16 cells showed a better stimulation of IL-1β production in the presence or absence of the inhibitors, indicating of up-regulation of these MAPKs.

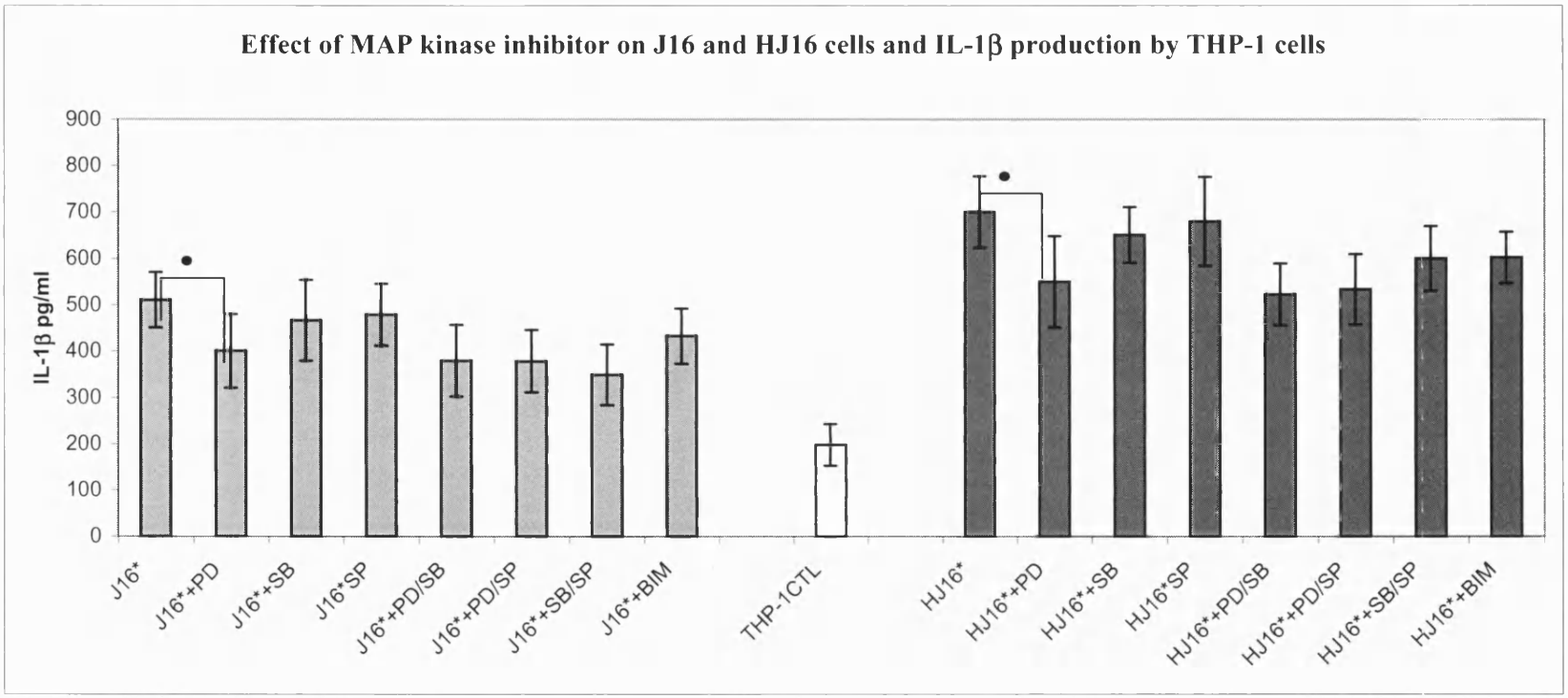


Figure 4.6: Effect of MAP kinase inhibitors on J16 and HJ16 cells and their stimulation of IL-1β production by THP-1 cells. A significant reduction in IL-1β production by THP-1 cells co-cultured with J16 or HJ16 cells treated with ERK1/2 inhibitor (PD98059), and the combination of SB203580 + SP600125. Treatment of J16 and HJ16 cells with SB203580, SP600125, and BIM resulted in a slight reduction in IL-1β production by THP-1 cells. (PD=PD98059 (20μM), SB=SB203580 (1μM), SP=SP600125 (20μM), PD+SB=PD98059+SB203580, PD+SP=PD98059+SP600125). The results expressed as the mean value of five experiments +/-SEM, (* activated cells with ionomycin and PDBu, ● indicates p value <0.05).

4.2.1 Effect of BSO and MAPK inhibitors on the J16 and HJ16 cells, and their stimulation of IL-1β production by THP-1 cells.

Redox regulation of MAPK–mediated cytokine biosynthesis has not been well characterized. However, it has been reported that pre-treatment with BSO facilitated TNF-α-induced MAPK p38 activation (Hashimoto et al; 2001). Previously in this thesis, it was shown that pre-treatment of J16 and HJ16 cells with BSO increased IL-1β production by THP-1 cells in co-culture (section 4.2). The effect of BSO and MAPK inhibitors on J16 cells was analysed.

Treated or untreated J16 cells (with BSO) were incubated with MAPK inhibitors for 1 hour, prior to activation for 24hours, and co-cultured with THP-1 cells with LPS. IL-1β production by THP-1cells was measured and the results were expressed as the mean value of three experiments.

Treatment of J16 and HJ16 cells BSO enhanced the production of IL-1β by THP-1 cells (Figure 4.7). However, PD98059 inhibitor significantly reduced IL-1β production ($p<0.05$) in the presence or absence of BSO. However, BSO treated cells showed slight increase in the stimulation of IL-1β production after ERK1/2 inhibitors compared to untreated cells.

BSO-treated J16 cells augmented IL-1β production in the presence or absence of SB203580, SP600125 and SB203580/SP600125 inhibitors ($p<0.05$).

From previous results, (Figure 4.2a) the levels of IL-1β from the co-culture supernatant was higher in BSO-pre-treated J16 and HJ16 cells, than control cells,

indicating that pre-treatment of BSO facilitated LPS/PDBu/ionomycin-induced IL-1β production. Since activated p38 MAP kinase is an important signal, which regulates cytokines production, the enhancement of p38, MAP kinase activation by BSO might result in the facilitation of IL-1β production. To test this possibility, cells that had been treated with BSO or NAC were stimulated with PDBu and LPS for 15 minutes. As shown in Figure 4.8, BSO enhanced p38 MAP kinase phosphorylation, after PDBu and LPS activation, and NAC had no effect on p38 activation.

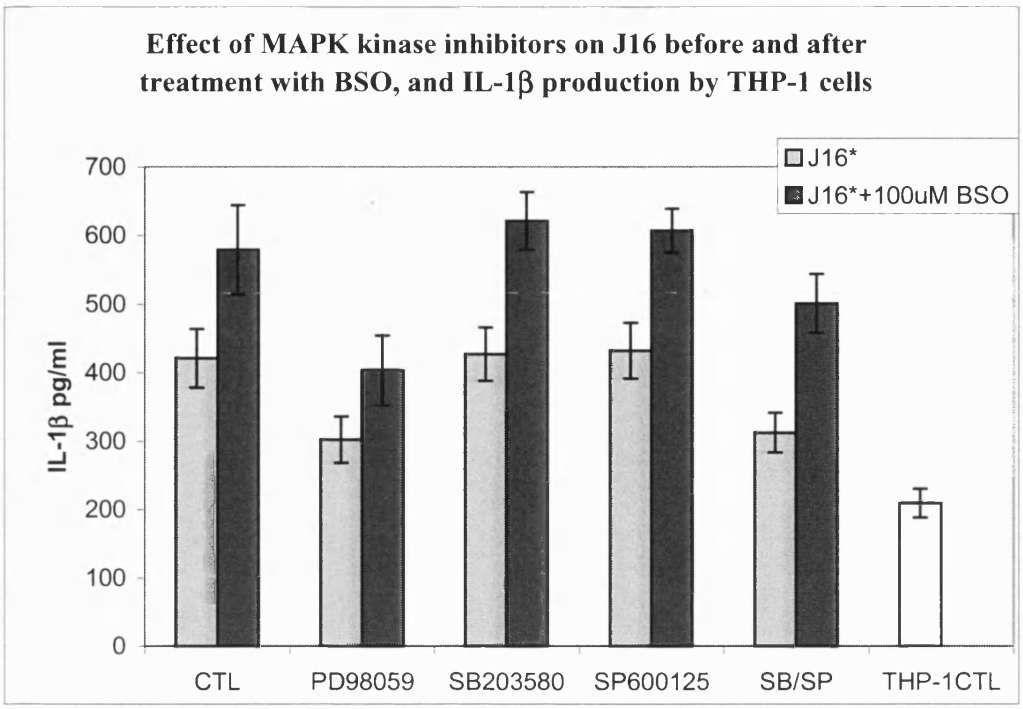


Figure 4.7: Effect of MAP kinase inhibitors on J16 before and after treatment with BSO and stimulation of IL-1β production by THP-1. BSO enhance the production of IL-1β in the presence or absence of MAPK inhibitors. PD inhibitor was potent in both treated and untreated cells. BSO enhanced IL-1β production in the presence or absence of SB203580, SP600125 and the combination of both SB203580 and SP600125. The result presented is the mean of three experiments, (+/-SEM), (* =activated cells with ionomycin/PDBu).

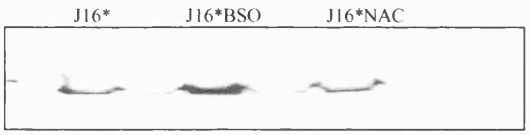


Figure 4.8: BSO up-regulates phosphorylated p38 MAP kinase activation, (with PDBu and LPS for 15 minutes), NAC had no effect. Two experiments gave similar result.

CHAPTER 5

RESULTS

THE EFFECT OF MODULATION OF GLUTATHIONE LEVELS ON CALCIUM SIGNALLING AND CELL PROLIFERATION OF J16, HJ16 AND UVA-R-J16 CELLS

Chapter 5 Results

5.1 The effect of modulation of glutathione on calcium signalling and cell proliferation of J16, HJ16 and UVAR-J16 cells

T cells in the inflamed joints of RA patients show features of hyporesponsiveness. This hyporesponsiveness is reflected by low proliferative responses, both *in vivo* and *in vitro*, as well as low calcium responses upon stimulation (Maurice et al; 1997).

Glutathione (GSH) is an important constituent of intracellular antioxidant defences, acting to reduce the toxic effects induced by free radicals. T lymphocytes isolated from the synovial fluid of RA patients contain decreased levels of GSH compared to T lymphocytes isolated from peripheral blood, (Carruthers et al; 1996, Maurice et al; 1997).

5.1.1 Intracellular calcium signalling in J16, HJ16 and UVAR-J16 cells

Intracellular calcium signalling in J16, HJ16 and UVAR-J16 was examined after stimulation of the cells with PHA-L (25 μg/ml), anti-CD3 (10 μg/ml), anti-CD28 (10 μg/ml), ionomycin (500nM) and hydrogen peroxide (50μM).

The peak and the plateau rise in intracellular calcium signal from baseline were calculated using a program provided with the system. All three cell types (J16, HJ16 and UVAR-J16) showed a rapid rise in intracellular calcium signal to a peak followed by a lower plateau signal (Figure 5.1). No significant differences were observed in the baseline of intracellular calcium signal of all three cell types. The range of values of the baseline of J16, HJ16 and UVA-J16 was between 15-50nM. For HJ16 and

UVAR-J16 cells the peak and the plateau was less than the peak and the plateau of J16 cells. There was a significant reduction ($p<0.05$) in both the peak rise and the plateau of HJ16 (the median of the peak 100nM for the plateau 40) and UVA-R-J16 cells (the median of the peak 125, and for the plateau 45 nM) compared to the J16 cells (the median of the peak 200 nM, and for the plateau 60) (Figure 5.1). This significant reduction in the peak and plateau of HJ16 cells intracellular calcium signalling was also seen in experiments involved different stimulants such as anti-CD3, (Figure 5.2), anti-CD28 (Figure 5.3), low concentration of ionomycin (500nM) (Figure 5.4), and H_2O_2 (Figure 5.5). Moreover, no significant difference was observed in the peak or the plateau of intracellular calcium signalling between PHA-L and anti-CD3 or anti-CD28 stimulation. Addition of low dose of ionomycin (500 nM) caused a sudden rise in the peak in a time less than 10 seconds after addition of ionomycin in both J16 and HJ16 cells, whereas, other stimuli gave peaks of intracellular calcium signal after 100-140 seconds after addition of the stimuli. As expected, the peak value (the median 450nM for J16 cells and 250 for HJ16 cells) obtained with ionomycin was significantly higher ($p<0.05$) than the peak value of intracellular calcium signal obtained with other stimuli (the median 200 for J16 and 100 for HJ16 cells). No significant differences were obtained in the plateau for all stimuli, except for H_2O_2.

No plateau of intracellular calcium signalling of both J16 and HJ16 cells was obtained after addition of H_2O_2. Addition of 50μM H_2O_2 resulted in a significant increase in intracellular calcium signalling of J16 cells compared to HJ16 cells, which showed no effect (Figure 5.5). This continuous increase in intracellular calcium signal in J16 cells was observed for 400 seconds, and no plateau was achieved. This may be due to an adverse effect of H_2O_2 on the cells. However, the highest value recorded of

intracellular calcium signalling of J16 cells (about 60nM after 400 seconds) was significantly less than those obtained with other stimuli.

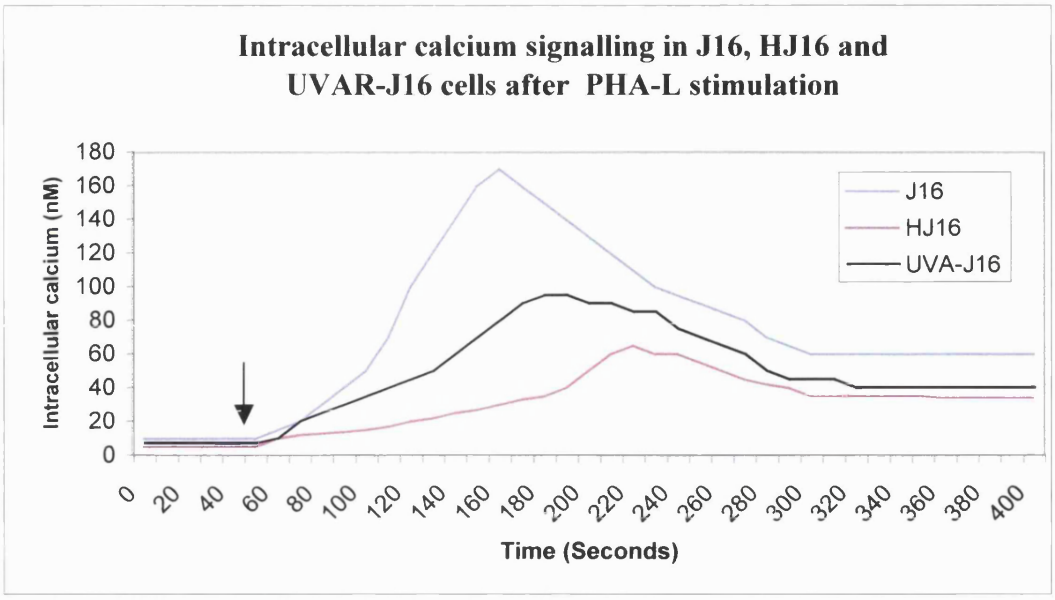


Figure 5.1: Representative example of intracellular calcium signal that were seen with J16, HJ16 and UVA-J16 cells loaded with Fura-2 and stimulated with 25μg/ml PHA-L (indicated as ↗) . The baseline, peak, and plateau of intracellular calcium were recorded on the PTI flourimetry system. A significant reduction in intracellular calcium was seen in HJ16 and UVAR-J16 cells compared to J16 cells. ($p<0.01$, statistic was done on the peak values of 10 experiments of J16 and HJ16 and 5 experiments of UVA-J16).

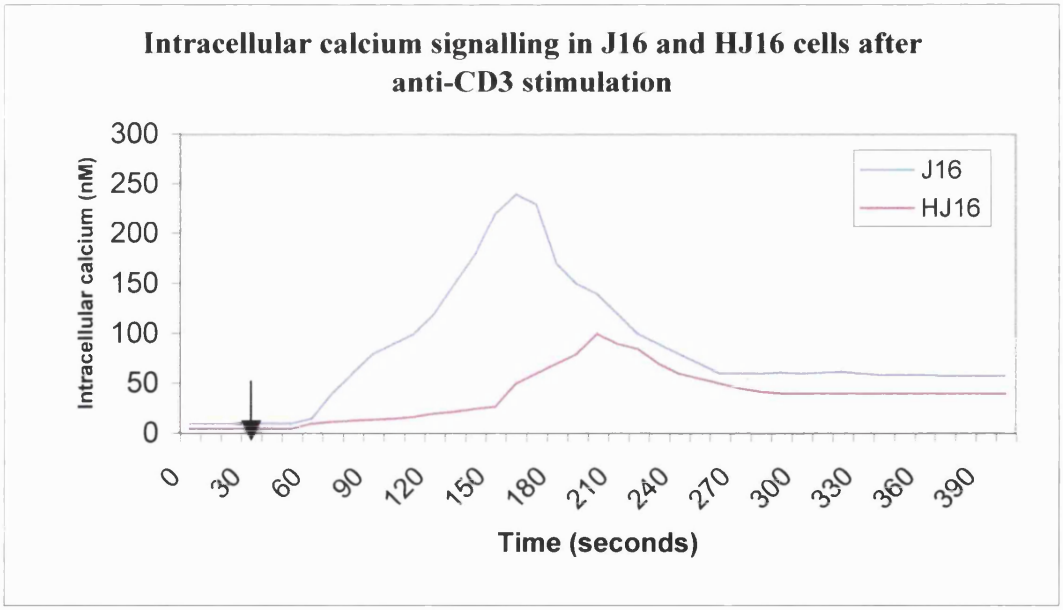


Figure 5.2: Representative example of the 5 patterns of intracellular calcium signalling in J16 and HJ16 cells after anti-CD3 stimulation. A significant reduction in intracellular calcium with HJ16 was recorded.

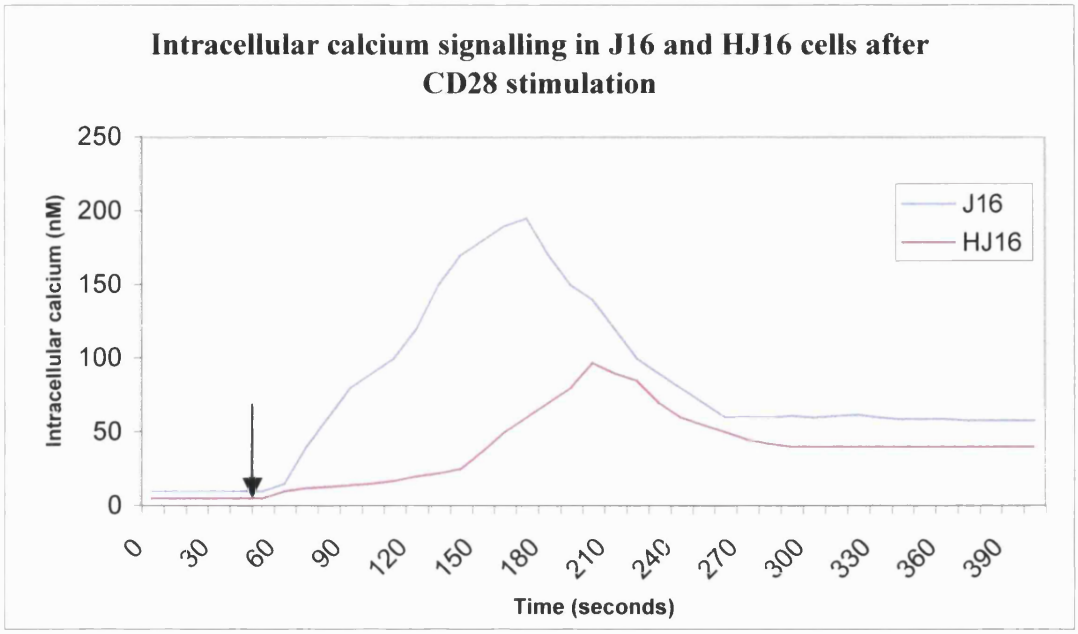


Figure 5.3: Intracellular calcium signalling in J16 and HJ16 after CD28 stimulation, HJ 16 exerted a reduced intracellular calcium signalling compare to J16. (Representative example of 5 patterns).

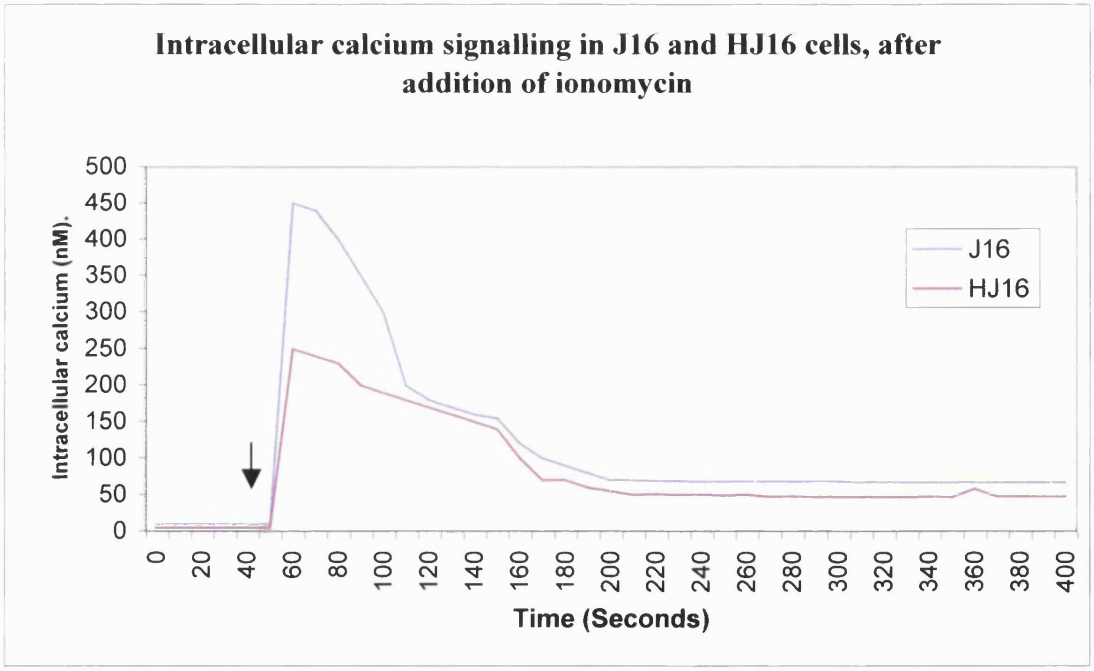


Figure 5.4: Intracellular calcium signalling in J16 and HJ16 after addition of ionomycin (500nM), HJ16 exhibited reduced intracellular calcium signalling compare to J16, (Representative example of 5 similar patterns).

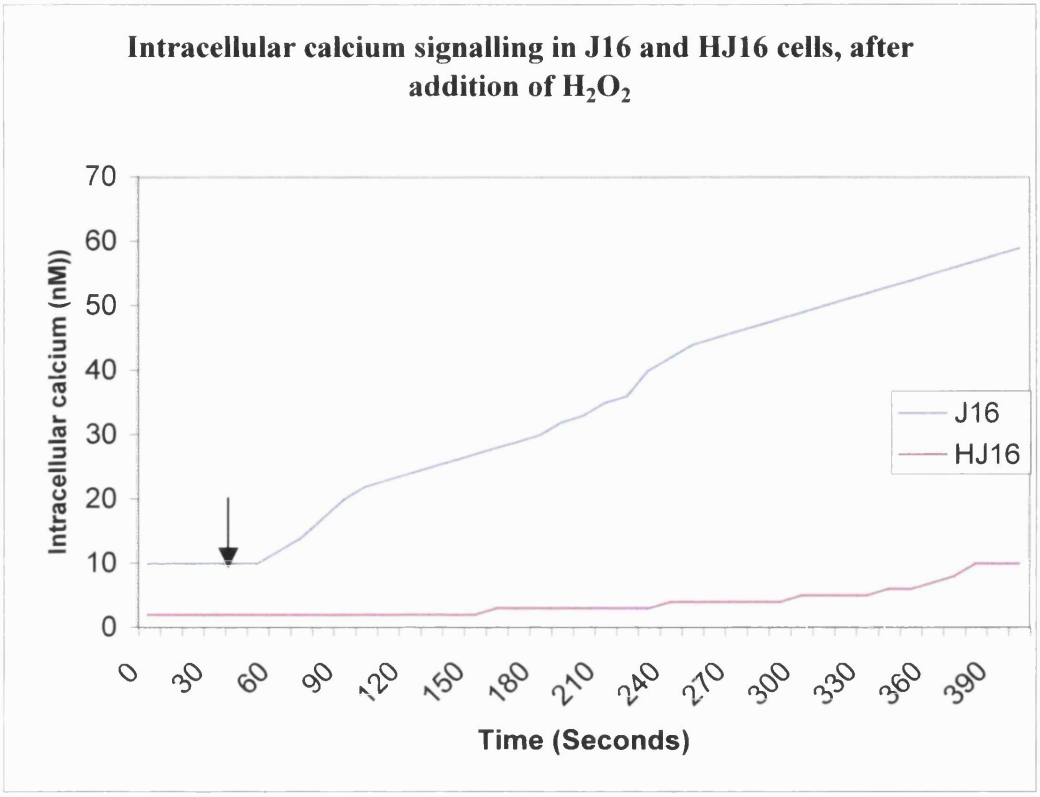


Figure 5.5: Effect of 50μM of hydrogen peroxide (H_2O_2) in intracellular calcium in J16 and HJ16 cells, no effect of H2O2 was seen in HJ16, intracellular calcium signalling was enhanced in J16, (Representative example of 3 similar patterns).

5.1.2 Investigation into the effect of depletion of GSH on intracellular calcium signalling

BSO, DEM and diamide are widely used to lower GSH levels, and micromolar concentrations of BSO decrease intracellular calcium responses after anti-CD3 stimulation (Staal et al; 1994). J16 and HJ16 cells were treated with various concentrations of BSO (25 - 200 μM), 100μM DEM and 100μm diamide. Experiments using trypan blue dye exclusion were always performed in order to exclude any overt toxicity of these drugs.

Treatment of J16 cells with 25-200μM of BSO resulted in a significant decrease in the peak and the plateau of intracellular calcium signalling (Figures 5.6a). No significant differences in the base line were observed. However the peak and the plateau of J16 cells treated with 200mM BSO was higher than the peak and plateau of J16 cells treated with 25-100μM BSO. This reduction in calcium signalling was best seen with 25-100μM BSO, ($P<0.05$). Slight reduction in intracellular calcium signalling of J16 was also observed with 200μM BSO. DEM and diamide treatment also resulted in significant decrease in calcium signalling in J16 ($p<0.05$) (Figure 5.7).

Depletion of GSH in HJ16 cells resulted in slight decrease in intracellular calcium signalling (Figure 5.6b). This slight reduction in intracellular calcium signal was seen in the base line, the peak and the plateau.

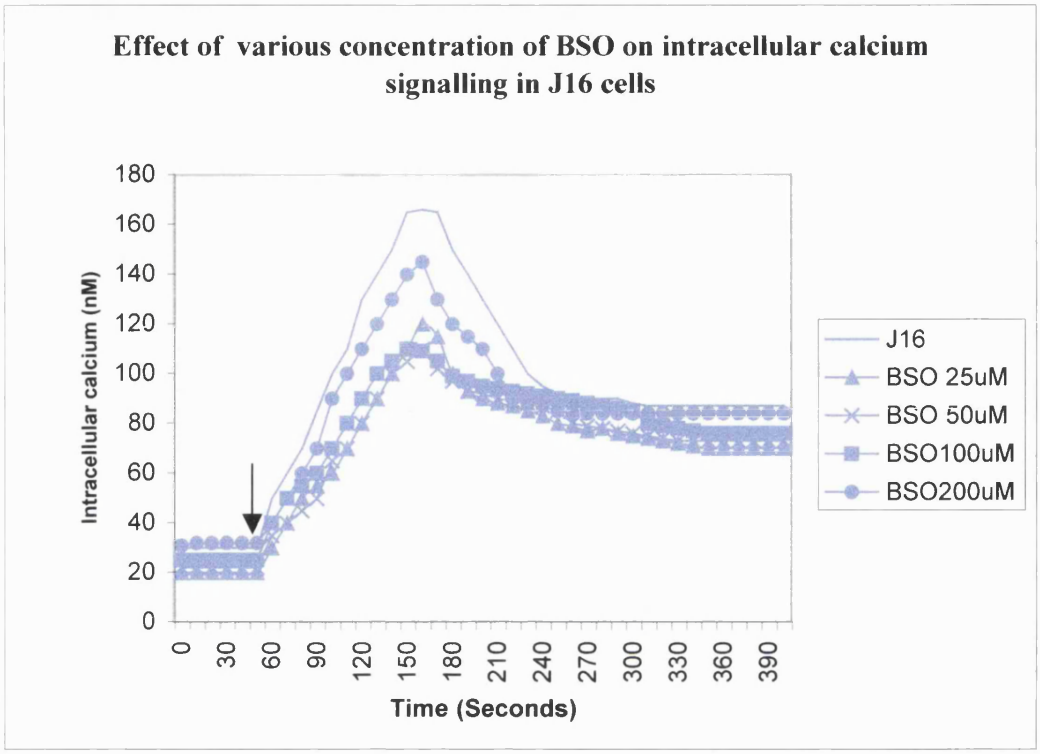


Figure 5.6a:GSH regulates the PHA-L-induced intracellular calcium in Jurkat cells (J16). PHA-L induced calcium flux is significantly decreased in BSO-treated Jurkat cells, (representative example of five similar experiments).

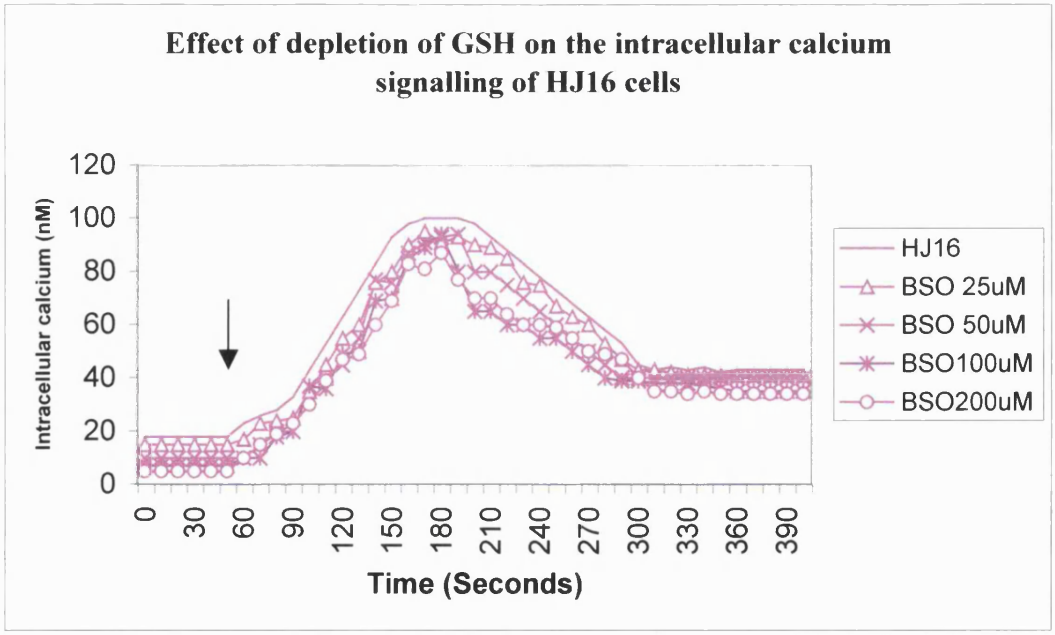


Figure 5.6b:GSH regulates the PHA-L-induced intracellular calcium in HJ16 cells. PHA-L induced calcium flux is insignificantly decreased in BSO-treated HJ16. Representative example of five similar experiments.

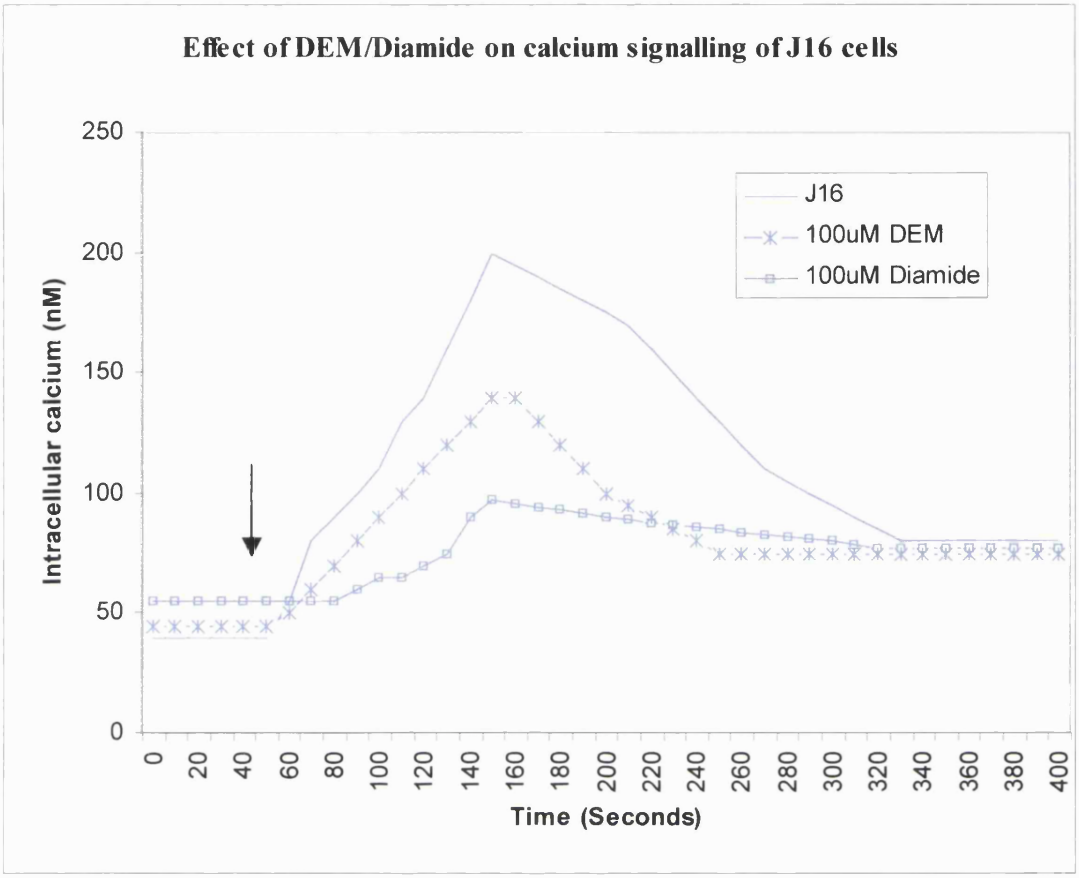
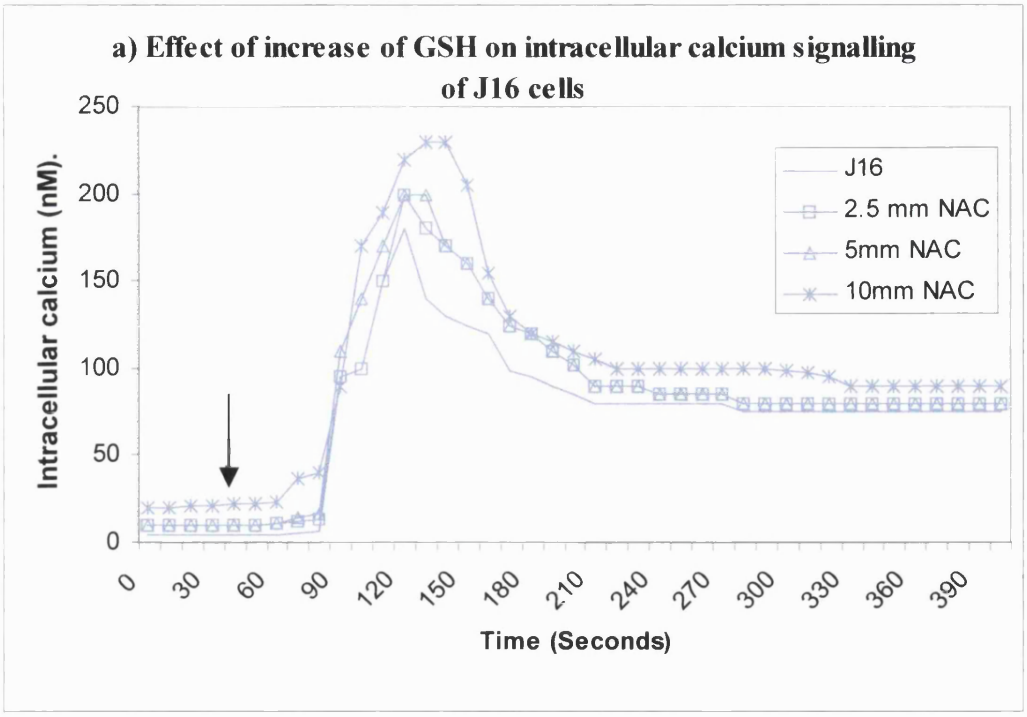


Figure 5.7 Treatment of J16 cells with 100μM DEM or diamide to deplete GSH resulted in decrease intracellular calcium signalling after PHA-L stimulation. (Representative example of three similar experiments).

5.1.3 Effect of N-acetylcysteine on intracellular calcium signalling

N-acetylcysteine (NAC) is a GSH precursor, so it has been used to enhance GSH in cells. Normal Jurkat cells (J16) and H_2O_2 tolerant cell line (HJ16) were treated with 2.5, 5, 10 mM NAC for 24 hours. PHA-L induced calcium flux was analysed in both J16 and HJ16 cells. Treatment of J16 cells with 10mM NAC enhanced the base line, peak, and the plateau of intracellular calcium signalling but not statistically significant. 2.5 and 5mM NAC had no significant differences in intracellular calcium signalling of J16 cells (Figure 5.8a). NAC treatment of HJ16 cells showed no significant differences in the base line, the peak, or the plateau of intracellular calcium signalling, compared to untreated cells figure 5.8b.



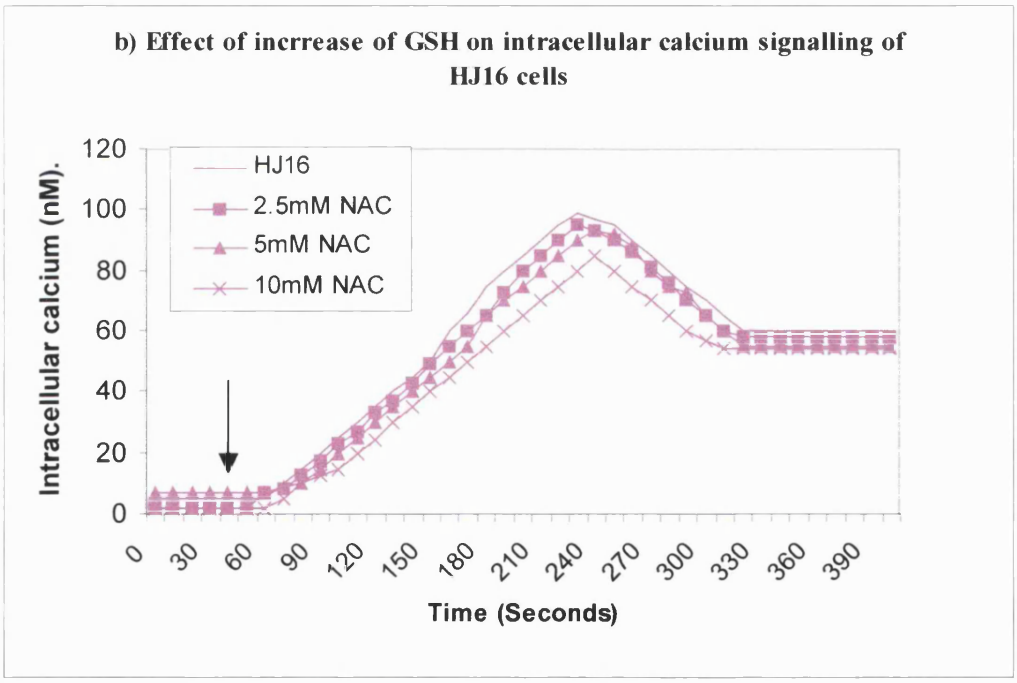


Figure 5.8:GSH regulates the PHA-L-induced intracellular calcium in (a) J16 and (b) HJ16 cells. PHA-L induced calcium flux is increased in J16 cells treated with 10mM NAC for 24hours, no significant differences were observed with other concentration in both J16 and HJ16 cells. Representative example of three similar experiments.

5.1.4 Investigation of proliferation of J16, HJ16 and UVAR-J16 cells and the effect of GSH levels

In order to investigate the proliferation rate in J16, HJ16 and UVAR-J16 cells and the effect of GSH levels, the XTT assay was performed to assess any differences in these cells. Briefly, cells were pre-treated with various concentrations of BSO/DEM/ diamide or NAC, washed and resuspended at $1x10^5$ cells/ml; 100 µl of each sample was plated into 96 well plates and incubated for 48 hours with various stimulants- anti-CD3 (10 µg/ml), PHA-L (10 µg/ml), ionomycin; (1 µM), PDBu, (1 µM), and a combination of ionomycin/PDBu. The results were expressed as the percentage of absorbance to J16 (as 100%).

In the control cells, a significant decrease in proliferation was seen with HJ16 cells and UVAR-J16 cells compared to normal J16 cells ($p<0.05$) (Figure 5.10). Activation of cells with anti-CD3, PHA-L, ionomycin, PDBu and ionomycin/PDBu markedly decreased the proliferation of all cells. However, PHA-L (10 µg/ml) activation promoted a significant reduction of proliferation in all three-cell types compared to other stimulants. This is clearly manifested with HJ16 cells ($p<0.01$), therefore, the effect of PHA-L on the proliferation was studied further, and low concentrations (0.1-10µg/ml) were used, (Figure 5.12). HJ16 cells were sensitive to low concentration of PHA (0.1 µg/ml). This was manifested by the decline in proliferation, which started at concentration of 0.1 µg/ml, whereas J16 cells at 1 µg/ml, so there is about 10 fold difference in the sensitivity of these cells.

Depletion of GSH with various concentrations of BSO (50, 100 and 200 μM), DEM (50, 100μM and 1mM) and diamide (50,100 μM, and 1mM) exerted markedly reduced proliferation, ($p<0.01$). No significant differences among these drugs or concentrations were seen, all resulted in significant reduction in the proliferations of J16, HJ16 and UVAR-J16 cells. NAC treatment (2.5, 5 and 10mM) showed a significant increase ($p<0.05$) in proliferation of J16 cells and much greater for HJ16 and UVAR-J16 cells (Figure 5.12).

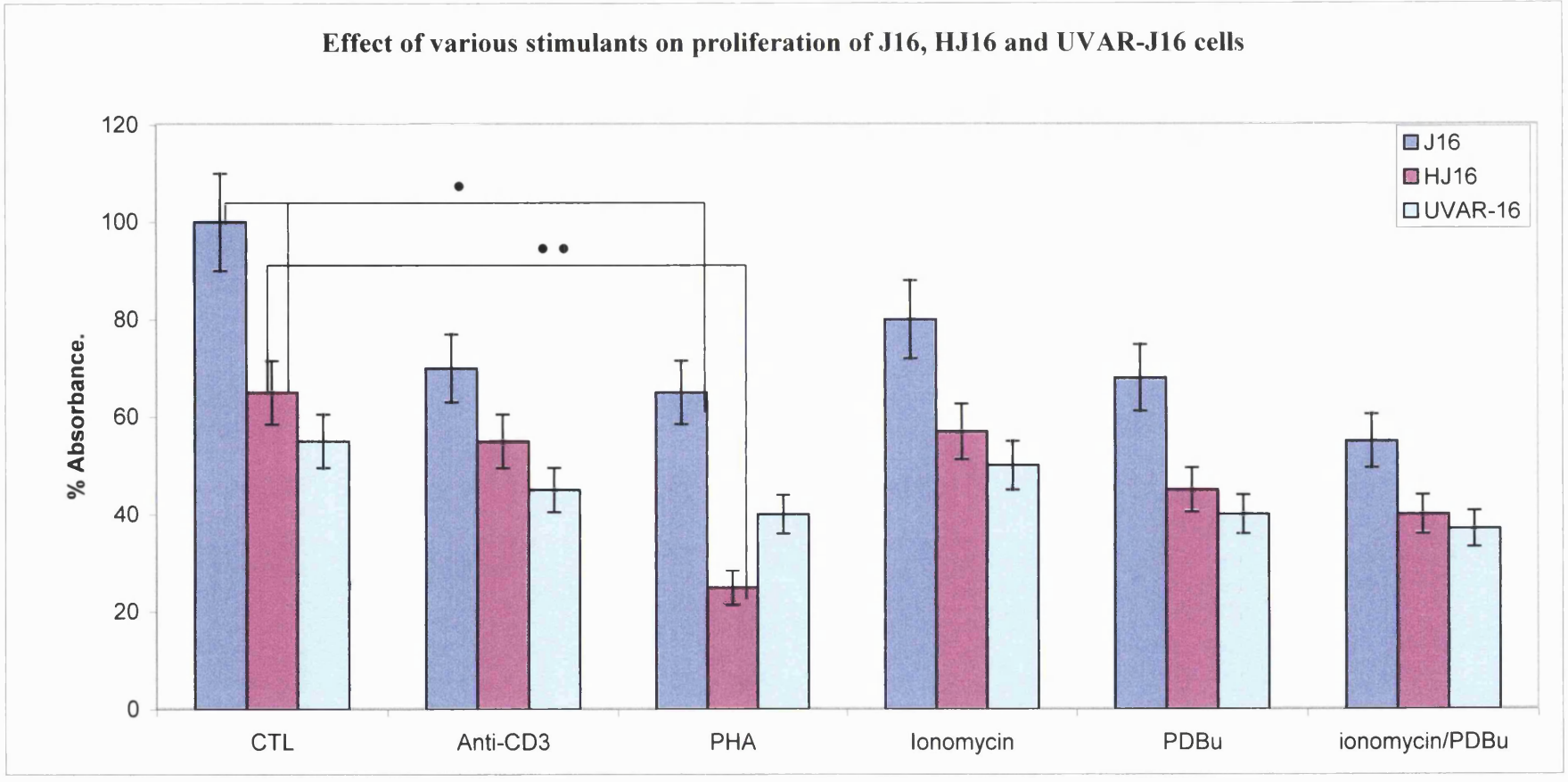


Figure 5.9: proliferation of J16, HJ16 and UVAR-J16. HJ16 and UVAR-J16 cells showed a substantial decrease in proliferation. Stimulation of cells with anti-CD3, PHA, ionomycin, PDBu, and ionomycin/PDBu caused reduction in proliferation of all three-cell lines. (The presented data is the mean of the absorbance of three experiments. • indicates $p<0.05$, or • • $p<0.01$.

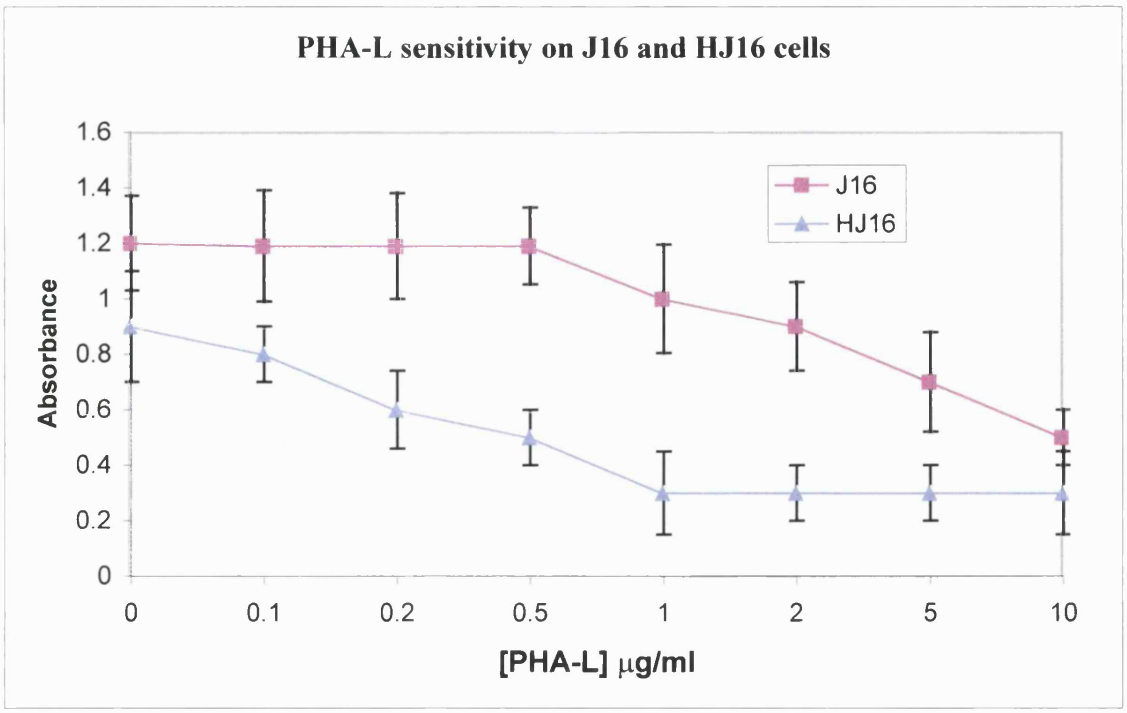


Figure 5.10 Effect of various concentrations of PHA-L on J16 and HJ16 cell proliferation. HJ16 cells were sensitive to 0.1 µg/ml PHA, whereas J16 cells at 1 µg/ml. Sensitivity are measured by reduction in proliferation of the cells. The data shown is the mean absorbance of three experiments (+/- SEM).

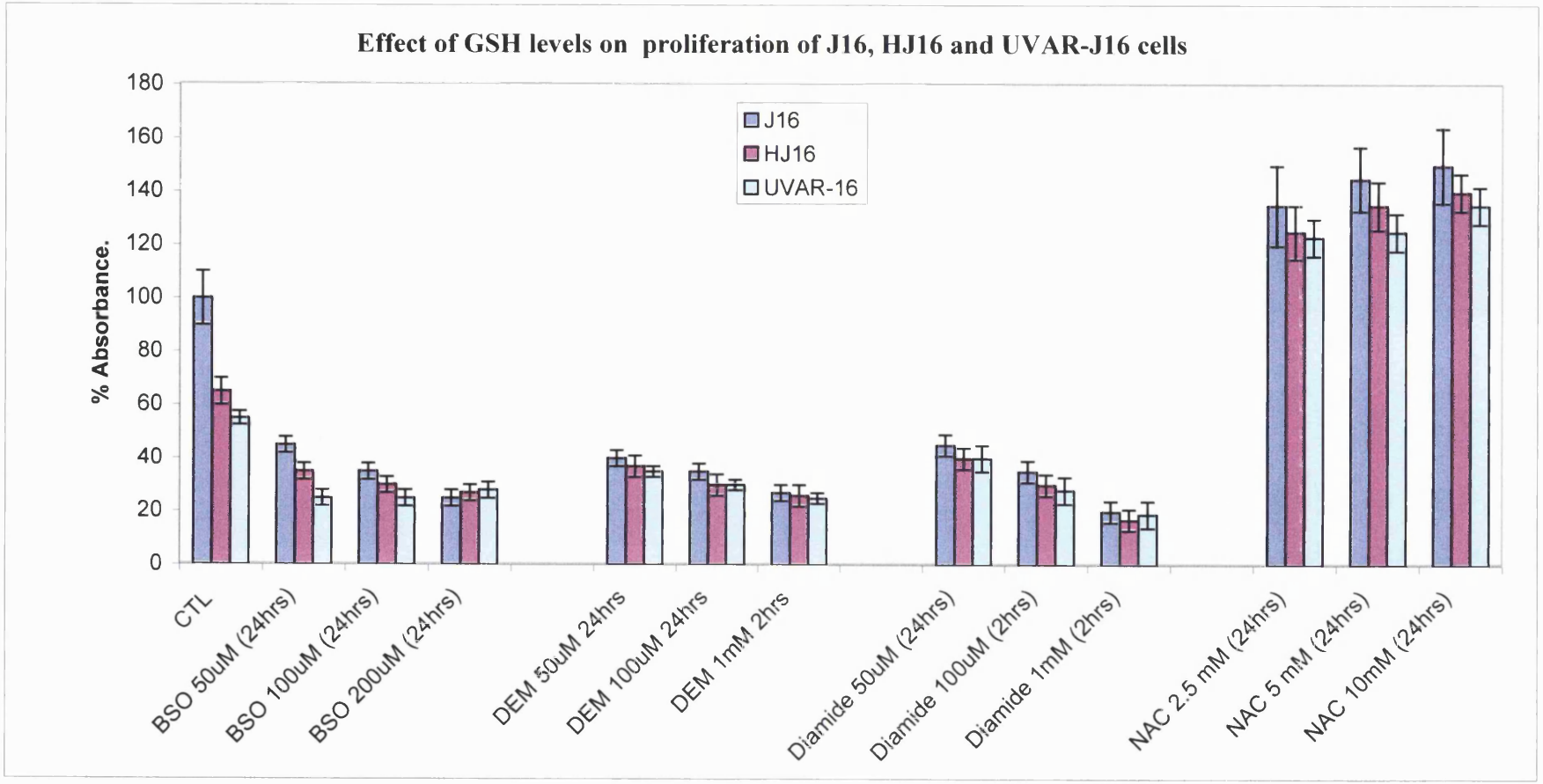


Figure 5.11: Modulation of GSH by BSO, DEM, diamide and NAC influences proliferation of J16, HJ16 and UVAR-J16. Depletion of GSH, with BSO, DEM or diamide, significantly reduced proliferation. Increase of GSH with NAC enhanced proliferation of J16 cells and significantly increases the proliferation of HJ16 and UVAR- J16 cells (The presented data is the mean of the absorbance of three experiments).

CHAPTER 6

DISCUSSION

Chapter 6 Discussion

6.1 Characterisation of a hydrogen peroxide tolerant T cell line (HJ16) in co-culture with monocytes

The results reported in chapter 3 showed that unfixed activated HJ16 cells and HJ16 clones were much more active in co-culture, in terms of stimulation of IL-1β production by PBMCs (Figure 3.7a) and THP-1 cells (Figure 3.10a), compared to J16 cells. Also, HJ16 cells were sensitive to low concentrations of PHA-L manifested by higher expression of CD2 and CD69 (Figure 3.5), stimulation of IL-1β production by THP-1 cells than J16 cells (Figure 3.13), and inhibition of proliferation (see section 6.3). Chronic exposure of J16 cells to oxidative stress (HJ16 cells) up-regulates complexes of proteins and transcription factors essential for cell activation and cytokine production or cell surface expression. PHA-L activates cells via CD2, and induces a high production of cell signalling molecules such as DAG (Pelassy et al, 1991). All unfixed activated T cells (J16, HJ16, and HJ16 clones) stimulated much higher level of IL-1β by both PBMCs and THP-1 cells, compared to fixed T cells, indicating soluble factors may be involved. Fixed activated T cells stimulated more IL-1ra production by THP-1 cells, but not by PBMCs.

The presence of hypoxia and oxidative stress in RA synovium has been well documented. Hypoxia favours proinflammatory cytokine production by monocytes, which may be due to an effect on T cells (Mapp et al, 1995). Our results with HJ16 cells, which have been exposed to oxidative stress (H_2O_2), are in good agreement with this observation. Under resting conditions, HJ16 and HJ16 clones (AB10, AD8, BA6 and BD3) showed slight activation, manifested by the expression of CD69, whereas

normal J16 cells did not show any activation (Figure 3.3a,b, and Figure 3.5a). Previous work by Isler et al showed that CD69 might be involved in the induction of proinflammatory cytokines (Isler et al, 1993). However, stimulation with PDBu alone resulted in up-regulation of CD69 (Figure 3.3a/b), but does not correlate with the stimulation of IL-1β and IL-1ra production by THP-1 cells in co-culture. No significant production of IL-1β and IL-1ra was obtained when THP-1 cells were cultured with J16 or HJ16 cells activated with PDBu alone (3.11a/b). This result provides no support for a CD69 mediated effect in the co-culture system. Further work is required to confirm this observation; Isler et al demonstrated that blocking of CD69 with antibodies could partially inhibit contact induction of cytokine release during co-culture, although other uncharacterised surface proteins are also required for contact activation (Isler et al, 1993). Recently, it has been found that high-density lipoprotein (HDL)-associated apolipoprotein A-I (apo A-I) inhibits cellular contact between T cells and monocytes, and consequently, inhibits IL-1β and TNF-α production (reviewed by Burger & Dayer, 2002).

As mentioned above, unfixed activated HJ16 cells and the clones induced significantly higher production of IL-1β by both PBMCs (Figure 3.7a) and THP-1 cells (Figure 3.10a) in the presence of LPS compared to J16 cells. This supports the hypothesis for a role of redox imbalance in the production of proinflammatory cytokines. These findings suggest that H_2O_2 induced changes in HJ16 cells, which in some way lead to higher induction of IL-1β. Cells respond to oxidative stress by a tightly controlled sequential process. The sequence of events begins with the sensing of the environmental changes at the cellular level via a signalling mechanism involving the extracellular matrix and cell surface sensor molecules. The sensor then

transduces the signal to the intracellular environment through second messenger systems that subsequently activate transcription factors (such as hypoxia inducible factor-1, Hif-1) and initiate specific transcriptional events. Moreover, it has been found that oxidative stress or hypoxia activates transcription factors such as NF-κB and AP-1, that are involved in the production of IL-1β. These transcription factors are directly subjected to redox regulation through their cysteine residues such that a reduced state in these cysteine residues is essential for their activity (reviewed by Ginn-Pease & Whisler, 1998; Kamata & Hirata, 1998; Haddad, 2002b). In addition, oxidative stress regulates certain protein tyrosine kinases and phosphatases, which may leads to the production of proinflammatory mediators. H_2O_2 rapidly induces substantial increases in c-jun and c-fos mRNA levels as well as increases in DNA binding of transcription factor AP-1 (reviewed by Kamata & Hirata; 1998). Moreover, oxidative stress such as exposure to H_2O_2 can rapidly induce marked increases in tyrosine phosphorylation in Jurkat cells (Whisler et al; 1995). Human T and B cells treated with millimolar concentration of H_2O_2 exhibit increased activity of protein-tyrosine kinase (PTK) (such as $p56^{lck}$ and $p72^{syk}$), MAPK and S6 peptide kinase (Whisler et al; 1995, reviewed by Kamata & Hirata; 1998). Thus exposure of HJ16 cells to H_2O_2 might influence cellular signalling pathways, and render them more active in co-culture.

All unfixed activated T cells stimulated more IL-1β production by PBMCs and THP-1 cells than fixed activated T cells. There are several explanations for this outcome: soluble factors other than IFN-γ or IL-17 (see below) may contribute to the production of such cytokines, hence, the supernatants of activated J16 and H16 cells showed a slight stimulation of THP-1 cells to produce IL-1β (Figure 3.12a), but this increase

was not statistically significant. This may require high concentration of the supernatants or more cells could be used. The effect of IL-17 on our co-culture system is excluded, because no IL–17 release by J16 or HJ16 cells was detectable.

Alternatively, T cells may mediate their effect through cellular contact rather than soluble factors. T cell cytokines such as IL-4, IL-10, IL-13 and transforming growth factor (TGF)-β have predominantly anti-inflammatory effects, IFN-γ and IL-17 display a weak activation capacity in terms of IL-1β and TNF-α induction, suggesting that soluble factors produced by T cells are not pathological mediators of RA. Therefore, T cells might exert a pathological effect through direct cellular contact with monocyte-macrophages (reviewed by Burger & Dayer, 2002; Dayer, 2003). In this work, fixed T cells were used to study the effect of cellular contact without soluble factors involved. However, fixation of T cells with glutaraldehyde is very artificial and may cause changes or alteration of cell surface molecules essential for the stimulation of IL-1β and IL-1ra production by PBMCs or THP-1 cells. Therefore, we do not exclude the effect of cellular contact with unfixed T cells co-cultured with PBMCs or THP-1 cells and stimulated IL-1β and IL-1ra production. It should be noted the co-culture was performed in 96 well U-shaped plates, where T cell and monocytes are in close contact. It has been documented that direct cell-cell contact between monocyte-macrophages and stimulated T cells can induce different patterns of cytokine production, depending on the T cell type and stimulus. This suggests that multiple cell surface molecules are involved in the contact-mediated activation of monocytes. Imbalance in the production of proinflammatory versus anti-inflammatory cytokines has also been observed. A study performed by Chizzolini et al (1997) demonstrated that cell membranes from antigen-stimulated, but not from resting, T

cells induced dose-dependent cytokine production by THP-1 cells, and Th1 cells induced higher levels of IL-1β production than Th2. In contrast, Th2 cells favour IL-1ra production by THP-1 cells, via direct cell-cell contact. A similar pattern was obtained in our study in that fixed T cells (Th2-like cells, see below) induced higher levels of IL-1ra than IL-1β by THP-1 cells, but not by fresh PBMCs. Moreover, Vey et al (1992) demonstrated that direct contact with stimulated T cells favours the production of IL-1β over that of IL-1ra in both PBMCs and THP-1 cells. In contrast, soluble factors release by stimulated T cells favour the production of IL-1ra (Vey et al, 1992). Our results showed that unfixed T cells stimulated both IL-1β and to lesser extent IL-1ra production by PBMCs. However, fixed T cells were better inducers for IL-1ra production by THP-1 cells but not by PBMCs. Thus it can be concluded that, direct cell-cell contact with activated T cells is a potent mechanism, which induces not only IL-1ra production but also proinflammatory cytokines such as IL-1β, TNF-α, IL-6 and MMPs (Vey et al, 1992; Isler et al, 1993; Lacraz et al, 1993). Several receptors and ligands have been implicated in mediating contact-dependent phenomena, including membrane-associated cytokines such as TNF-α, LFA-1/ICAM-1, CD40/CD40L, lymphocytes activation antigen (LAG-3), CD69 and CD2/LFA3 (Wagner et al, 1994; reviewed by Burger & Dayer, 2002; Dayer, 2003). CD69 and CD2, however, are up regulated on HJ16 cells compared to J16 cells, before and after activation with PHA-L (Figure 3.5). Up-regulation of such molecules (other than CD69) may explain why HJ16 cells were more active in terms of stimulation of IL-1β and IL-1ra production by PBMCs and THP-1 cells. The significant reduction in the production of IL-1β by both PBMCs (Figure 3.7b) and THP-1 cells (Figure 3.10b) cells after fixation of T cells may be due to negative affects of glutaraldehyde on surface molecules that are essential in the stimulation of IL-1β production.

It has been shown that T cell subsets (Th1, Th2) may determine the type of cytokines that can be produced by monocytes (Chizzolini et al, 1997). Thus, we attempted to classify J16 and HJ16 cells and the clones by using chemokine receptors (Figure 3.6). The results revealed that J16, HJ16 and the clones are perhaps more Th2–like. Further confirmation is required for this classification. Moreover, there was no apparent difference in the expression of CCR2, CCR3, CCR4, CCR5 and CXCR3 at mRNA level between J16 and HJ16 cells or the clones. However the percentage of $CD3^+$ lymphocytes expressing CCR2, CCR4, CCR5, and CXCR1 was significantly elevated in RA peripheral blood compared with that in normal peripheral blood, while the percentage of $CD3^+$ lymphocytes expressing CCR5 was significantly enhanced in RA synovial fluid compared with that in normal and RA peripheral blood (Ruth et al, 2001).

Many co-cultures of T cell and monocyte cell lines (THP-1 cells) or PBMCs were carried out in the presence of LPS. LPS has profound effects on the production of cytokines (IL-1β and IL-1ra) by monocytes-macrophages. Interestingly, it has been demonstrated that T lymphocyte activation by LPS is strongly dependent on direct cell-cell contact of responding T lymphocytes with viable accessory monocytes (Mattern et al, 1994), mainly represented by interaction of B7 with CD28 (Mattern et al, 1998). Therefore, the use of LPS in our co-culture system may provide co-stimulation to the T cell line (J16 and HJ16 cells) as well as directly stimulating the monocyte cell line (THP-1 cells) or PBMCs.

The ratio of IL-1β to IL-1ra was approximately 1:15 at all treatments. Endogenous production of IL-1ra is an important anti-inflammatory mechanism both in animal models and in human disease. In the rheumatoid synovium, an imbalance exists in this system, such that levels of IL-1ra are not adequate to effectively block the proinflammatory effects of IL-1 (IL-1ra binds to IL-1RI with an avidity equal to that of IL-1), a high level of IL-1ra is essential to reduce the effect of IL-1β. *In vitro* experiments have revealed that an excess of 100 times the amount of IL-1ra is required to inhibit IL-1β activity, whereas, *in vivo*, 100-2000 times more IL-1ra is needed (Arend, 1993; Firestein et al, 1994; reviewed by Arend; 1996/2001).

The pattern of IL-1ra production was different in THP-1 cells and PBMCs. Fixed J16, HJ16 cells and HJ16 clones resulted in higher production of IL-1ra by THP-1 cells, but not by PBMCs (Figure 3.10d) in the presence of LPS compared to unfixed cells (Figure 3.10c). Fixed HJ16 cells and the clones induced higher IL-1ra production than fixed J16 cells, although this was not statistically significant. This may be due to the binding efficiency of these cells. It has been reported that exposure of Jurkat cells to H_2O_2 increased the binding to THP-1 cells. The membrane binding sites on the oxidized Jurkat cells were found to be sialylated poly-N-acetylgalactosaminyl sugar chains (Beppu et al, 2000). The difference in the patterns of IL-1ra generation obtained with THP-1 cells and PBMCs may also be due to the difference in the response to LPS. LPS-induced expression of the human sIL-1ra gene in macrophages is controlled by at least two PU.1 (a transcription factor) binding sites. The proximal PU.1 site, binds both NF-κB and GA-binding protein (GABP). This site overlaps the previously identified NF-κB binding site and thus represents a novel composite NF-κB/PU.1/GABP binding site. Mutation that disrupted either NF-κB or PU.1 had no

effect on the LPS response. However, a mutation that disrupted both NF-κB and PU.1-binding resulted in a greater than 50% decrease in the LPS response. Mutation of the distal site resulted in an approximately 50% decreased in the activation of the promoter in response of LPS. Thus LPS-induced regulation of sIL-1ra gene expression is a complex event potentially involving the interaction of three different transcription factors (NFκB/PU.1/GABP) (Smith et al, 1998). Expression of these receptors and transcription factors on THP-1 cells (which are biologically different from fresh PBMCs) and PBMCs might be different, which could explain why a different pattern of IL-1ra was obtained. Furthermore, it has been found that the rate of internalisation of LPS by THP-1 cells slowed significantly after a few minutes of exposure to LPS (Kitchens & Munford, 1998). Furthermore, LPS has been shown to initiate multiple intracellular signalling events, including the activation of NF-κB, which ultimately lead to the synthesis and release of a number of proinflammatory mediators, including IL-1, IL-6, TNF-α and IL-8 (Zhang et al, 1999). Similarly LPS might have a different effect on IL-1ra inducing-pathways on both THP-1 and PBMCs. It has been shown that molecules involves in LPS binding site are complicated and depends on the cell type, for example, LPS binding site on B cells has been shown to engage TLR-4, RP105 and MD1, whereas in mast cells the binding site is found to be TLR-4 and MD2 (reviewed by Triantafilou M & Triantafilou K, 2002). This provides further support to the hypothesis that the response of cells, such as monocytes and THP-1 to LPS may be heterogeneous and the interaction of a group of transcription factors with a single *cis*-acting element may depend on the relative affinities and the concentration of proteins as well as their localization within the nucleus (Arend et al, 1995; Smith et al, 1998).

The production of IL-1β (Figure 3.15a) and IL-1ra (Figure 3.15b) by PBMCs increased significantly after exposure to 2% O_2 in the presence of LPS and slightly in the absence of LPS, indicating that co-stimulus is required to enhance IL-1β or IL-1ra generation. This is in agreement with previous studies showing that hypoxia induces IL-1 release in mononuclear cells stimulated by LPS (Ghezzi et al, 1991; Naldini et al, 2001). The potentiation of IL-1β and IL-1ra production by 2% O_2 indicates a mechanism of regulation of cytokine production by hypoxia. This mechanism could be important in many pathologic conditions. A significant reduction in the production of IL1β and IL-1ra by PBMCs co-cultured under 1% O_2, this may be due to that 1% O_2 was lethal to the cells and affected their viability.

No significant difference was found between J16 and UVA-R J16 cells in their stimulation of IL-1β and IL-1ra production by THP-1 cells (Figure 3.14). This indicates that UVAR-J16 cells are biologically different to HJ16 cells, and different oxidative stresses may result in different responses. Further investigation of the effects of oxidative stress caused by H_2O_2 and UVA on Jurkat cells is needed to explain why UVAR-J16 cells are similar to J16 in co-culture, whereas, HJ16 cells are more active. Further work is being undertaken by other researchers in our laboratory to explore the nature of HJ16 cells, UVAR-J16 and the effect of redox balance in the activation of transcription factors such as NF-κB.

6.2 Manipulation of the intracellular antioxidant, glutathione (GSH) in J16 and HJ16 cells and their stimulation of IL-1β and IL-1ra production

The contribution of redox imbalance, which often results from enhanced ROS formation, to the molecular events that lead to the inflammatory response, is not well understood. Cells possess several antioxidants or reductants that maintain the intracellular redox environment in a highly reduced state. Glutathione (GSH) is a major cellular reductant, involved in the elimination of ROS such as H_2O_2. Recently accumulating evidence has indicated that cellular redox plays an essential role not only in cell survival, but also in cellular signalling systems (redox regulation) (reviewed by Kamata & Hirata, 1998; Haddad, 2002b).

HJ16 cells displayed certain characteristics not associated with RA synovial fluid cells. HJ16 cells exhibit increased GSH level compared to normal J16 cells (Figure 4.1), whereas synovial T cells have depleted GSH (Maurice et al, 1998). Moreover, it has been demonstrated that levels of GSH are reduced in erythrocytes from RA patients (Chilles et al, 1990), and that reduced GSH may be responsible for the differentiation of T cells towards a Th1 phenotype (Peterson et al, 1998). This difference in GSH levels in HJ16 cells and RA synovial fluid T cells may be due the acute and chronic exposure to oxidative stress. HJ16 cells have been exposed to a chronic oxidative stress resulted in high GSH level, whereas RA synovial fluid T cells are in subjected to acute oxidative stress continuously (Mapp et al, 1995).

Interestingly, BSO, DEM, and diamide treatment to deplete GSH in J16 and HJ16 cells enhanced the stimulation of IL-1β and IL-1ra production by THP-1 cells

(Figures 4.2 and 4.4). It is not clear whether the up-regulation of IL-1ra production was due to a direct effect of depletion of GSH on T cells or is due to the increase of IL-1β levels. Further investigation is required to determine the effect of GSH levels on IL-1ra production. In contrast, NAC treatment resulted in a slight reduction in the stimulation of IL-1β and IL-1ra production (Figure 4.3). Our results, in agreement with previous studies on epithelial cells (Haddad, 2001), showed that depletion of GSH with BSO augmented proinflammatory cytokine production. In vitro the regulatory effect of BSO on proinflammatory cytokine production has been studied. Growing evidence implicates an association between redox signalling and the regulation of proinflammatory cytokine production, thereby placing more importance on the utilization of intracellular GSH. The GSH biosynthesis is blocked by BSO, a specific inhibitor of γ-glutamylcysteine synthetase. Consequently, the capacity of T cells (J16 and HJ16 cells) to replenish intracellular stores of GSH is dramatically affected, thereby modulating the equilibrium necessary to evoke a defence strategy in pathophysiology (Hayes & McLellan, 1999). Depletion of GSH is associated with generation of ROS, leading to augmentation of proinflammatory cytokine production. This may be mediated at least in part by the activation of redox-sensitive transcription factors such as NF-κB (Ginn-Pease & Whisler, 1998, Kamata & Hirata, 1999). The pathway mediating BSO-induced up-regulation of pro-inflammatory cytokines by T cells is a complex process and involves several mediators. BSO is capable of inducing intracellular accumulation of ROS, GSH is involved in H_2O_2 reduction (a major source of $OH^{\cdot}$) in epithelial cells, and GSH depletion results in H_2O_2 accumulation and increased $OH^{\cdot}$ production (Hayes & McLellan, 1999).

Furthermore, GSH depletion in murine APCs decreased the secretion of IL-12 and led to polarization from the typical Th1 cytokine profile towards Th2 response patterns, suggesting that GSH levels in APCs play a central role in determining whether a Th1 or Th2 cytokine response predominates (Peterson et al; 1998). It has been reported that IL-12 production is regulated by the redox potential of murine peritoneal macrophages. ''Reductive'' macrophages with elevated GSH levels produced IL-12, whereas ''oxidative'' macrophages with a reduced level of GSH did not (Murata et al, 2002).

On the other hand, NAC treatment of J16 and HJ16 cells resulted in a slight reduction in IL-1β production by THP-1 cells (Figure 4.3), although this reduction is not statistically significant. On the other hand, there is no enhancement of IL-1β and IL-1ra production. Thus NAC treatment has an effect, which is opposite to that of BSO-treated cells. It has been reported that NAC reduced LPS-mediated secretion of TNF-α and IL-6 and blocked the phosphorylation/activation of MAPK p38 in epithelial cells (Haddad, 2002c). The effect of NAC on cytokine production is still controversial. A recent study suggested that NAC-dependent regulation of IL-1β is tightly regulated by NF-κB, and showed elevation in IL-1β production following incubation with NAC and LPS (Parmentier et al, 2000). Other investigators, however, have reported an antagonist effect of NAC on proinflammatory cytokine biosynthesis and NF-κB regulation (Ginn-Pease & Whisler, 1998, Verhasselt et al, 1999). We cannot exclude the possibility, however, that a NAC-mediated inhibitory effect on soluble factors released by NAC-treated J16 or HJ16 cells may be linked with the IκB/NF-κB pathway.

The synovium of RA is hypoxic and contains several different cell types, including macrophages and T cells; these cells are in close contact. The hypoxic nature of the synovium alters the intracellular GSH levels. It has been found that synovial T cells have depleted intracellular GSH levels (Maurice et al, 1997). As mentioned above T cells might exert a pathological effect through direct cellular contact with monocyte-macrophages. BSO treatment of J16 and HJ16 cells may enhance the expression of cell surface molecules involved in contact mediated monocyte activation, as well as their counter-ligands. It has been postulated that T cell membrane-associated TNF-α may be involved in macrophage activation. Hence, depletion of GSH with BSO (cellular thiol status) and oxidative stress (H_2O_2) promote NF-κB activation, and may also up-regulated membrane-associated molecules on J16 or HJ16 cells, consequently enhancing cellular contact and cytokine production. Moreover, it has been found that depletion of intracellular GSH increased neutrophil adhesion to human umbilical vein endothelial cells via up-regulation of ICAM-1, P-selectin, and E-selectin. NAC treatment attenuated this adhesion (Kokura et al, 1999).

In addition, MAPK pathways are also sensitive to GSH levels and contribute to the increase in cytokine production. It has been shown that BSO facilitated TNF-α induced p38 MAP kinase activation in human epithelial cells (Hashimoto et al; 2001). Our preliminary results showed up-regulation of phosphorylated p38 after BSO treatment of J16 cells (Figure 4.8). MAPKs in HJ16 cells and in BSO treated J16 and HJ16 cells seem to be up-regulated, hence the effect of MAPK or PKC inhibitors (PD98059, SB203580, SP600125, and bisindolylmaleimide) had less effect on these cells compared to J16 or untreated cells (Figures 4.6 and 4.7). It is well documented that cellular redox changes are involved in the activation of the MAPK superfamily

(reviewed by Kamata & Hirata, 1998). Our results are in agreement with previous observations. MAPKs (p38, ERK1/2, and JNK) are likely to play a critical role in the pathogenesis of RA, for example JNK enhances transcriptional activity of AP-1, a key regulator of MMP production. IL-1 is a potent inducer of JNK phosphorylation. Initially, it was believed that JNK and p38 pathways alone were responsible for mediating responses to inflammatory cytokines. These kinases act in part by phosphorylation and activating the transcription factors c-Jun and ATF2. Upon activation, these transcription factors can induce the promoters of multiple genes, including those for MMPs. In contrast ERK1/2 were thought to be responsible for transducing growth factor-dependent proliferative signals. However, chemical inhibition of the ERK1/2 pathway with PD98059, (which inhibit MEK1 and MEK2, two enzymes that phosphorylate and activate ERK1/2) has raised additional issues: it has been shown that when fibroblast-like synovial cells are treated with this compound and stimulated with serum, phorbol ester, or growth factors, the proliferative responses are blocked, as well as activation of MMP-1. Inhibition f these kinases in T cells seem to block the stimulation of IL-1β production by co-cultured monocytes (Rincon et al, 2000; Han et al, 2001; reviewed by Vincenti, et al 2001). Our results showed inhibition of IL-1β production after incubation of J16 or HJ16 cells with ERK1/2 inhibitors (PD98059), JNK inhibitor (SP600125), p38 inhibitor (SB203580), and PKC inhibitor (bisindolylmaleimide). This confirms the broader roles of these MAPKs (especially ERK1/2), at least in cultured cells.

6.3 The effect of modulation of glutathione on calcium signalling and cell proliferation

The results presented in chapter 5 demonstrated that HJ16 cells compared to J16 cells have a reduction in the peak and plateau of intracellular calcium signalling in response to PHA-L (Figure 5.1), anti-CD3 (Figure 5.2), anti-CD28 (Figure 5.3), and even ionomycin (Figure 5.4). A similar pattern has been observed in synovial fluid (SF) and peripheral blood T cells from patients with RA compared to normal controls (Allen et al, 1995, Carruthers et al, 1996 & 2000). A change in the pattern of the intracellular signal in cells may influence subsequent activation events at the nuclear level, thus affecting the functional outcome of such stimuli, including cytokine production. T cell activation requires sustained threshold intracellular calcium, and the necessary level varies from cell to cell (reviewed by Berridge et al, 2000). However, reduction in intracellular calcium signalling does not correlate with stimulation of IL-1β and IL-1ra production by PBMCs and THP-1 cells in the co-culture system reported in chapter 3. However, it does correlate with the proliferation assay reported in chapter 5.

Oxidative stress has an effect on the cellular metabolism and structural elements of cells; such effects can change the calcium signal and modify essential pathways. It has been reported that oxidants such as H_2O_2 causes a rapid increase in calcium concentration in the cytoplasm of diverse cell types (Wang & Joseph, 2000). Addition of low concentration of H_2O_2 to J16 cells, but not to HJ16 cells resulted in continuous increase in intracellular calcium (Figure 5.5). It seems that this increase can be due to both calcium release from internal cellular stores such as the ER/SR, and calcium import from extracellular spaces. Similarly, Roveri et al observed that mild oxidative

stress caused by moderate H_2O_2 levels transiently increased cytoplasmic calcium levels even in calcium free medium, but failed to do so if calcium was first depleted from the ER/SR (Roveri et al 1992).

There are several possible explanations as to why HJ16 cells have a reduction in intracellular calcium signalling: Oxidants can directly attack membranes forming lipid peroxides, which can activate phospholipase A2 (PLA2). The products of PLA2 actions (arachidonic acid and hydroxyl fatty acids) are known as mediators of such important physiological processes essential for immune responses and inflammation. It has been demonstrated that lipid peroxides alter calcium homeostasis, which in turn can modulate PLA2 activity in endothelial cells (Elliott et al, 1992). In addition, elevated calcium levels in the cytoplasm can induce other enzymes that produce free radicals such as nitric oxide synthase (Lynch & Dawson, 1994). Thus, by elevating cytoplasmic calcium levels, oxidants can also indirectly cause more oxidant production and further escalate calcium levels. HJ16 cells were subjected to alteration in the membrane forming lipid and intracellular enzymes and proteins essential for calcium binding (buffering system). For example calcineurin activity (an important enzyme in calcium dependent eukaryotic signal transduction pathways) is sensitive to cellular redox state, it has been found that H_2O_2 can completely abrogate calcineurin-mediated NF-AT trans-activation in response to stimulation (Reiter et al, 1999; reviewed by Berridge et al, 2000). Further investigation of the calcium binding proteins is required to elicit their nature in HJ16 cells.

IP3 which is the recognised second messenger releasing calcium from intracellular stores (ER/SR), may induce a second independent effect by enhancing calcium influx

through plasma membrane permeable channels. Once initiated, the process leads to the activation of PKC and to a wide variety of cellular responses, which culminate in cytokine production and cell proliferation (see below). Oxidants, however, also can regulate production of IP3 and calcium release from ER/SR as a consequence (Doan et al, 1994). These stores in HJ16 cells may have depleted levels of calcium due to exposure to H_2O_2. Moreover, H_2O_2 can effectively inhibit calcium transport by sarco-endoplasmic reticulum calcium transporting ATPases (SERCA), in smooth muscle (Grover et al, 1992). SERCA are ATP-dependent calcium pumps that transport calcium against its concentration gradient into the lumen of ER/SR, thus antagonising the function of IP3 by reducing free cytoplasmic calcium levels and accumulating calcium in ER/SR. It has been found that SERCA can be inhibited by oxidation of its sulphydryl groups and also by direct effect of oxidants on the ATP binding site (Castilho et al, 1996, Xu et al, 1997). Furthermore, hypoxia alters Na^{2+}/Ca^{2+} exchangers (essential in equilibrium of calcium in the mitochondria) causing more calcium to be transported into mitochondria instead of its release from mitochondria (Griffiths et al, 1998).

It can be concluded that oxidants such as H_2O_2 and duration of exposure could dramatically change calcium signalling and even reverse its direction from stimulative to repressive (or vice versa). Hence chronic exposure to H_2O_2 rendered HJ16 cells with reduced calcium signalling, and addition of H_2O_2 to J16 cells increased calcium signalling. However, addition of H_2O_2 to J16 cells resulted in continuous increase in calcium signalling and no peak or plateau was obtained, this may be due to membrane damage caused by H_2O_2.

The intracellular GSH concentration in T cells provides mechanisms for modulating responses elicited by antigens and cytokine signals. Depletion of GSH in J16 cells resulted in a significant decrease in intracellular calcium signalling (Figure 5.6a), but only a slight decrease with HJ16 cells was observed (Figure 5.6b). GSH depletion induces an imbalance in the antioxidant defence, including the activity of such enzymes as superoxide dismutase, catalase and glutathione peroxidase. Glutathione peroxidase for example has been found to be decreased significantly after BSO treatment (Thanislass et al, 1995). Diminished GSH concentration affects the regulation of reductive process by the GSH redox pathway (Reed, 1986). Moreover, protein-thiols are essential for a number of membrane functions, such as enzyme activities (e.g. Na, K ATPase) and the transport system. It has been shown that some protein thiols are essential for calcium transport in mitochondria and microsomes and that loss of such protein thiols can lead to perturbations of cellular calcium homeostasis (Kramer et al, 1987). In addition, the sulfhydryl groups are involved in calcium retention and their oxidation causes the depression of calcium sequestration in liver microsomes and mitochondria. The loss in calcium retention appears to be related to alterations in the status of GSH, which in turn, affects the permeability mechanisms (Thanislass et al, 1995). This could be a possible reason for the observed decreased intracellular calcium signalling after GSH depletion, but confirmation requires further study.

The rates of proliferation of the HJ16 and UVAR-J16 cells are significantly reduced compared to J16 cells (Figure 5.9), which may be due to exposure to oxidative stress and reduced calcium signalling in these cells as mentioned above. Calcium is one of the key regulators of cell proliferation (reviewed by Berridge et al, 2000). However,

all cells (J16, HJ16, and UVAR-J16 cells) showed a high proliferation rate in resting conditions. In normal T cells the induction of proliferation is dependent on the co-stimulatory signals (Suthanthiran et al, 1990), however our results showed that treatment of J16, HJ16, and UVAR-J16 cells with anti-CD3, PHA-L, ionomycin, PDBu, and combination of ionomycin and PDBu resulted in a reduction in proliferation of all cells (Figure 5.9). This reduction was seen most dramatically with HJ16 cells stimulated with PHA-L. HJ16 cells were more sensitive to PHA-L stimulation (Figure 5.10). This is not only due to up regulation of CD2 (PHA-L receptors) expression, but may be due to up regulation of intracellular molecules essential in cell signalling via CD2 or other PHA-L binding receptors. In normal T cells, anti-CD2 mAb alone failed to induce cell proliferation (Sunthanthiran et al, 1990). It has been documented that oxidative stress induces T cell hyporesponsiveness, manifested by impaired expression of some TCR-proximal signalling molecules such as TCR-ζ, p56, LAT (Cermerski et al, 2003) and ZAP-70 (Chakravarti & Abraham, 2002). Signalling through the CD2 molecules was shown to employ similar signalling molecules as the TCR (Bonin et al, 1998), thus exposure of J16 cells (HJ16 cells) to H_2O_2, may have had an effect on these molecules that consequently affected the CD2 signalling pathway.

Pretreatment of J16, HJ16, and UVAR-J16 cells with BSO, DEM, and diamide dramatically inhibited cell proliferation, and no significant differences between these cells were observed. This permits the interpretation that depletion of GSH in these cells is associated with a profound inhibition of their proliferative potential. Previous observations showed that decreased intracellular levels of GSH in T cells affected

both calcium responses and proliferation after anti-CD3 stimulation (Suthanthiran et al, 1990; Staal et al, 1994; Maurice et al, 1997).

In contrast to the effect of BSO on cell proliferation, NAC treatment increased the proliferation of J16, HJ16 and UVAR-J16 cells, maybe due to enhancement of GSH levels. It has been found that GSH enhanced T cell proliferation activity and increased IL-2 production (Liang et al, 1989). This suggests that the effect of GSH on the replication of activated T lymphocytes is partially, if not mainly, due to its influence on the production of IL-2. GSH may interact with IL-2 directly by reducing the disulfide bond of IL-2. However, this explanation is unlikely since the reduced form of IL-2 is less potent than the non-reduced form. This lead to an alternative explanation; GSH may affect the IL-2 activity indirectly through its effect on the target cells (Liang et al, 1989; Park et al, 2000). Blockade of surface SH groups on T cells causes a marked inhibition of mitogen-induced cell proliferation, due to down-regulation IL-2 receptors and interference with IL-2 binding to high-affinity receptors complex (Smith et al, 1992).

From the discussion above, it can be concluded that the intracellular GSH concentration in T cells performs a central role in modulating responses elicited by antigen and cytokine signals. At higher concentrations of GSH, proliferation will be favoured, whereas at lower concentrations of GSH, inflammatory-type responses that involve the induction of transcription factors NF-κB and AP-1 are more likely to occur. That is, when GSH levels are high, T cells respond poorly to stimulation in terms of calcium signalling and proliferation. In contrast, when GSH levels are low (after treatment with BSO), T cells fail to flux calcium or to proliferate. Instead, they

respond strongly in co-culture, with changes in cellular protein phosphorylation and induction of transcription factors such as NF-κB and AP-1.

In addition, long-term exposure to oxidants might lead to chronic changes in the delicate balance of the pro-oxidant and antioxidant status of the T cell, and consequently, counteract the initial effects of oxidative stress. Depletion of antioxidants may have a role in the development of autoimmune diseases such as RA. Treatment of RA patients with potential aliphatic thiols with strong reducing properties has been used for a long time, and shown to be beneficial (Halliwell et al, 1988).

6.4 SUMMARY AND CONCLUSION

Expression of CD69 showed that HJ16 and the clones are activated in resting conditions, presumably due to the exposure to H_2O_2. A slight up-regulation of CD2 was also seen with HJ16 cells in resting conditions and significantly after PHA-L activation compared to J16 cells.

Unfixed activated HJ16 cells and HJ16 clones significantly induced IL-1β production by PBMCs and THP-1 cells in the presence of LPS, compared to normal J16 cells. All unfixed and activated T cell lines (J16, HJ16, and clones of HJ16 cells) enhanced the production of IL-1β by both PBMCs and THP-1 cells, indicating soluble factors may be involved, as well as cell-cell contact. Fixed T cell lines promoted the production of IL-1ra by THP-1 cells but not by PBMCs.

Depletion of GSH in J16 and HJ16 cells augmented their stimulation of IL-1β and to lesser extent IL-1ra production by THP-1 cells. Enhancement of GSH levels in J16 or HJ16 cells had no effect on the stimulation of IL-1β or IL-1ra production by THP-1 cells.

HJ16 cells exhibited low intracellular calcium signalling and low proliferative rate. Depletion of GSH decreased intracellular calcium signalling and proliferation of both J16 and HJ16 cells. Increased GSH levels in J16 and HJ16 cells had no significant effect on intracellular calcium signalling, but significantly increased the proliferation of these cells.

In addition, the intracellular GSH concentration in T cells provides a central role for modulating responses elicited by antigen and cytokine signals. At higher concentration of GSH (after NAC treatment, but not after exposure to oxidative stress), proliferation will be favoured, whereas, at lower concentrations of GSH, an inflammatory-type response is more likely to occur. Moreover, when GSH levels are low, T cells fail to flux calcium or to proliferate. Instead, they respond strongly to inflammatory cytokine stimulation with changes in cellular protein phosphorylation and induction of transcription factors.

These results suggest a possible role of hypoxia and the interaction between T cells and monocytes in the rheumatoid synovium, which leads to the enhanced production of proinflammatory cytokines and perpetuation of inflammation.

6.5 FUTURE DIRECTION

ROS accumulation in J16 and HJ16 cells can be measured before and after treatment with BSO or NAC, with effect on MAPK and transcription factors (such as NF-κB) essential for cytokine production and cell surface molecules expression and their ligands.

Cell-cell contact is a potent mechanism, which induces both anti-inflammatory and pro-inflammatory cytokines. Several receptors have been shown to be involved in this including, CD40, CD40L, CD11b-CD18, and CD11c-CD18, CD23, CD69, LFA-1 and others. These molecules can be investigated on HJ16 cells.

Further investigation of the T cells and soluble factors (other than IL-17) involved in the co-culture could be highly productive. Modulation of antioxidants (with BSO or NAC) on soluble factors released by T cells or monocytes can be studied.

Moreover, calcium signalling in HJ16 cells and the BSO-treated T cell line was significantly reduced, molecules involved in calcium signalling such as IP3 and calcium-binding proteins can be investigated in HJ16 cells and related to chronic exposure to oxidative stress.

Finally, the different patterns of IL-1ra achieved by THP-1 cells and fresh PBMCs can be studied further. This may involve cell surface molecules expressed on THP-1 cells but not PBMCs or *vice versa*.

APPENDIX 1

Appendix 1

Culture Media, solutions and Buffers

RPMI 1640

Milli-Q water	400 ml
RPMI (10x)	50 ml
FCS	50 ml
Penicillin (1000IU/ml/Streptomycin (1000 µg/ml)	5 ml
Sodium Bicarbonate (7.5% w/v)	15 ml
L-Glutamine (200 mM)	5 ml
Soudium Hydroxide (10 M)	800 µl

Phosphate Buffer Saline (PBS)

NaCl	70.1 g
NaH2PO4.H2O	4.4 g
Na2HPO4.H2O	12.8 g
KCL	2 g
Milli-Q-water	10 litres

Alternatively, for tissue culture work, 5 tablets (Oxoid) in 500 ml Milli-Q water.

ELISA Solutions

Coating buffer: 0.5 M carbonate-bicarbonate buffer, pH 9.6.

Blocking buffer: 1% BSA, 5% sucrose in PBS, pH 7.4.

Wash buffer: 0.05% Tween 20 in PBS, pH 7.4.

Tris diluent: 0.1% BSA, 20 mM Trisma base (2.42 g/l), 150 mM NaCl (8.76 g/l), pH 7.3.
Stop solution: 0.5 M Sulphuric Acid.

Proliferation assay

Phenazine methosulfate (PMS): 1.53 mg/ml in PBS (5 mM stock solution stable at 4C^{o} for 3 months), protect from lighlt.

XTT: 1 mg/ml in RPMI medium, make up fresh at time of assay, dissolve at 37C^{o}

XTT-PMS:
To 1 ml of the XTT mixture, add 5 µl of PMS stock.

SDS-PAGE gel solutions (western blot)

5x TBE Buffer

Tris base	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8)	20 ml
Milli Q water to	1000 ml

Blue Juice

Bromophenol blue	50 mg
Ficoll 400	3 g
Milli Q water	20 ml

Reservoir Buffer

25 mM Tris	3.03 g
192 mM glycine	14.4 g
0.1% SDS (Lauryl sulfate)	1 g
Milli-Q water	1000 ml

Semi-dry transfer buffer

48 mM Tris	5.81 g
39 mM glycine	2.93 g
SDS	0.375 g
Methanol	200 ml
Milli-Q water	800 ml

Wash/Blocking buffer (TBS-T, 10x) (pH 7.5)

10 mM Tris	12.11 g
100 mM NaCl	58.44 g
0.1% Tween-20	10 ml
Milli-Q water	990 ml

For blocking add 5% non-fat powder or 1% BSA

4x Resolving gel buffer (pH 8.8)

1.5 M Tris	90.86 g
0.4% SDS	2 g
Milli-Q water	500 ml

4x Stacking gel buffer (pH 6.8)

0.5 M Tris	30.29 g
0.4% SDS	2 g
Milli-Q water	500 ml

Resolving gel (10%)

Milli-Ro water	8.17 ml
Resolving gel buffer (pH 8.8)	5 ml
Bis-Acryl (30%)	6.67 ml
10% AMPS	150 µl
TEMED	15 µl

Stacking gel (5%)

Milli-Ro water	6.85 ml
Stacking gel buffer (pH 6.8)	3 ml
Bis-Acryl (30%)	2 ml
10% AMPS	150 µl
TEMED	10 µl

Lysis Buffer (pH 7.5)

20 mM Tris	0.242 g
137 mM NaCl	0.801 g
NaF (500 mM stock)	2 ml
1mM EDTA100 mM stock	1 ml
1% Glycerol	10 g
1% NP-40 (IGEPAL)	1 g
Milli-Q water	make up to 100 ml

The following reagents must be added immediately prior to use:

1 mM sodium orthovanadate (500 mM stock)	0.2 µl/ml
1 mM PMSF (500 mM stock in DMSO)	2 µl/ml
Aprotinin 2 mg/ml stock	2 µl/ml
Leupeptin 2 mg/ml stock	2 µl/ml
Pepstatin A 5 mg/ml stock	0.4 µl/ml

2x Sample buffer (pH 6.8)

125 mM Tris	1.51 g
4% SDS	4 g
10% Mercaptoethanol	10 ml
20% Glycerol	20 g
0.04 % Bromophenol	0.04 g
Milli-Q water	make up to 100 ml

Reagents for measurement and manupilation of GSH

10% TCA

TCA	50 g
Milli-Q water	500 ml

5% TCA/2nM EDTA

10% TCA	5 ml
Milli-Q water	4.96 ml
EDTA (0.5 M)	40 µl

0.1M Phosphate buffer 1mM EDTA

Milli-Q water	224.5 ml
Potassium phosphate (monobasic)	9 ml
Potassium phosphate (dibasic)	16 ml
0.5 M EDTA	0.5 ml

(Filter in the hood)

DTNB (prepare fresh each time of experiment)

DTNB	3 mg
0.5% sodium bicarbonate ($NaHCO_3$)	2 ml

Dissolve at 37°C for 20 minutes, and sonicate in water bath sonicator for another 20 minutes, protect from light.

GSH reductase

0.1M Phosphate buffer 1mM EDTA	2 ml
GSH reductase (stock)	17.3 µl

β-NADPH: prepare a stock solution of 5 mM in 0.5% $NaHCO_3$, protect from light and store at –20°C.

Buthionine-DL-sulfoximine (BSO), diethyl Maleate (DEM), and diamide: prepare fresh at 50 mM working solution with RPMI.

N-acetylcysteine (NAC) 100mM working solution (prepare fresh each time of experiment)

NAC	0.086 g
RPMI	5 ml
Sodium bicarbonate	5 ml (to give pH 7.4)

Propidium iodide: 1mg/ml (Milli-Ro water).

BIBLIOGRAPHY

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Aaravak T., Chabaud M., Miossec P., and Natvig J.B., (1999). IL-17 is produced by some proinflammatory Th1/Th0 cells but not by Th2 cells. J. Immunol., **162**: 1246-1251.

Abe T., and Takeuchi, (2001). Rheumatoid arthritis and tumor necrosis factor α. Autoimmun., **34**: 291-303.

Abrahams V.M., Cambridge J., Lydyard P.M.L., and Edwards J.C.W., (1998). Fc-gamma RIIIα mediates tumour necrosis factor alpha secretion by human monocytes. Arthritis Rheum., **41**: 1873 (abstract).

Abramson S, and Amin A., (2002). Blocking the effect of IL-1 in rheumatoid arthritis protects bone and cartilage. Rheumatol., **41**: 972-980.

Abramson V. and Gallin J.I, (1990). IL-4 inhibits superoxide production by human mononuclear phagocytes. J. Immunol, **144**: 625-630.

Acuto R., and Michel F., (2003). CD28-mediated co-stimulation: A quantitative support for TCR signalling. Nature, **3**: 939-951.

Ahmed S. T., and Ivashkiv L.B., (2000). Inhibition of IL-6 and IL-10 signalling and stat activation by inflammatory and stress pathway. J. Immunol, **165**: 5227-5237.

Akashi S., Nagai Y., Ogata H., Oikawa M., Fukase K., Kusumoto S., Kawasaki K., Nishijima M., Hayashi S., Kimoto M., Miyake K., (2001). Human MD2 confers on mouse Toll-like receptors 4 species-specific lipopolysaccharide recognition. Int. Immunol., **13**: 1595-1599.

Akashoshi T., Oppenheim J., and Matsushima K., (1988). Interleukin-1 stimulates its own receptor expression on human fibroblasts through the endogenous production of prostaglandin(s). J. Clin. Invest., **82**: 1219-1224.

Allard S.A., Muirden K.D., Camplejohn K.L., and Maini R.N., (1987). Chondrocyte-derived cells and matrix at the rheumatoid cartilage-pannus junction identified with monoclonal antibodies. Rheumatol. Int., **7**: 153-159.

Allen M.E., Young S.P., Michell R.H, and Bacon, P.A, (1995) Altered T lymphocytes signalling in rheumatoid arthritis. Eur. J. Immunol, **25**:1547-1554.

Alvaro-Gracia J.M., Zaifler N.J. and Firestein G.S., (1989). Cytokines in chronic inflammatory arthritis. J. Exp. Med., **170**: 865-875.

Anderson K.C., Morimoto C., Paul S.R., Chauhan D., William D., Cochran M., (1992). Interleukin-11 promotes accessory cell-dependent B-cell differentiation in humans. Blood, **80**: 2797-2804.

Anderson M.E., (1998). Glutathione: an overview of biosynthesis and modulation. Chemico-Biological interactions, **111-112**: 1-14

Arend W., Smith M., Janson R., and Joslin F., (1991). IL-1 receptor antagonist and IL-1β production in human monocytes are regulated differently. J. Immunol., **147**: 1530-1536.

Arend W., (1993) Interleukin-1 receptor antagonist. Advan. Immunol, **54**: 167-227.

Arend W.P., and Dayer J.M., (1995). Inhibition of the production and effects of Interleukin-1 and tumour necrosis factor-α in rheumatoid arthritis. Arthritis Rheum., **38**: 151-160.

Arend W.P., Malyak M., Guthridge C., and Gabay C. (1998). Interleukin-1 receptor antagonist: Role in Biology. Ann. Rev. Immunol, **16**: 27-55.

Arend W.P., (2001). Cytokine imbalance in the pathogenesis of rheumatoid arthritis: the role of interleukin-1 receptor antagonist. Semin. Arhtitis Rheum., **30**: 1-6.

Arend W.P., (2001). Cytokines and cellular interactions in inflammatory syovitis. J. Clin. Invest, **107**: 1081-1082.

Arias-Negrete S., Keller K. and Chadee K., (1995). Proinflammatory cytokines regulate cyclooxygenase-2 mRNA expression in human macrophages. Biochem. Biophy. Res. Comm., **208**: 582-290.

Arkwright P.D., Mcdermott M.F., Houten S.M., Frenkle J., Waterham H.R., Aganna E., Hammond L.J., Mirakian R.M.Tomlin P.I., Vijaydurai P.I., & Cant A.J., (2002). Hyper IgD syndrome (HIDS) associated with in vitro evidence of defective monocytes TNFRSFIA shedding and partial response to TNF receptor blockade with etanercept. Clin. Exp. Immunol., **130**: 484-488.

Attur M., Patel R., Abramson S., and Amin A., (1997). Interleukin-17 up-regulation of nitric oxide production in human osteoarthritis cartilage. Arthitis Rheum., **40**: 1050-1053.

Attur M., Dave M., Cipolletta C., Kang P., Goldring M., Patel I., Abramson S., and Amin A., (2000). Reversal of autocrine and paracrine effects of interleukin-1 (IL-1) in human arthritis by type II IL-1 decoy receptor. J. Biol. Chem., **275**: 40307-40315.

Baeten D., Boots AM., Steenbakkers PG, Elewaut D, Bos E.S, Verheijden G.F., Berherijden G., Miltenburg A.M., Rijnders A.W, Veys E.M de Kesyser F.,(2000). Human cartilage gp-39+, CD16+ monocytes in peripheral blood and synovium: Correleation with joint destruction in rheumatoid arthritis. Arthritis Rheum., **43**:1233-1243.

Baeuerle P.A., and Henkel, T., (1994). Function and activation of NF-kappa-B in the immune system. Ann. Rev. Immunol., **12**: 141-179.

Barbulescu K., Becker C., Schlaak J.F., Schmitt, E., Zumbuschenfelde, K.H.M., and Neurath M.F., (1998). Cutting Egde: IL-12 and IL-18 differentially regulate the transcriptional activity of the human interferone-gamma promoter in primary CD4+ T lymphocytes. J. Immunol, **160**: 3642-3647.

Baumann H., & Schendel P., (1991). Interleukin-11 regulates the hepatic expression of the same plasma protein genes as interleukin-6. J. Biol. Chem., **266**: 20424-20427.

Berg W., and Chvatchko Y, (2001). Therapeutic effect of neutralizing endogeneous IL-18 activity in the collegen-induced model of arthritis. J. Clin. Invest., **108**: 1825-1832.

Berridge M.J., Lipp P., and Bootman M.D., (2000). The versatility and universality of calcium signalling. Nature, **1**: 11-21.

Berry N., Ase K., Kikkawa U., Kishimoto A., and Nishizuka Y., (1989). Human T cells activation by phorbol esters and diacylglycerol analogs. J. Immunol, **143**: 1407-1413.

Black R.A., Kronheim S.R., Sleath P., (1989). Activation of Interleukin-1β by a co-induced protease. FEBS Lett., **247**: 386-390.

Blair H.C., and Athansou N.A., (2004). Recent advances in osteoclast biology and pathological bone resorption. Histol. Histopathol., **19**: 189-199.

Blake D.R., Merry P., Unsworth J., Kidd B.L, Outhwaite J.M, Ballard R., Morris C.J, Gray L. and Lunec J., (1989). Hypoxic-reperfusion injury in the inflamed human joint. Lancet, **1**: 289-293.

Blom A.B., van Lent P.L., van Vuuren H., Holthuysen A.E., Jacobs C., van de Putte L.B., de Winkel J.G., and Berg W.B., (2000). FcγR expression on macrophages is related to severity and chronicity of synovial inflammation and cartilage destruction during experimental immune-complex-mediated arthritis (ICA). Arthitis Res., **2**: 489-503.

Bodamyali T., Stevens C.R., Billingham M.E.J, Ohta S., Blake D.R., (1998). Influence of hypoxia in inflammatory synovitis. Ann. Rheum. Dis., **57**: 703-710.

Bondeson J., Browne K.A., Brennan F.M., Foxwell B.M.J., and Feldmann M., (1999). Selective regulations of cytokines induction by adenoviral gene transfer of I kappa B proinflammatory cytokines are inhibited but IL-10 is nuclear factor independent. J. Immunol., **162**: 2939-2945.

Bonin V., Ehrlich, and Fleischer, (1998). The transmembrane region of CD2-associated signal-transducing proteins is crucial for the outcome of CD2-mediated T cell activation. Immunol., **93**: 376-383.

Boots A.M.H., Verheijden G.F.M., Schoningh R., van Staveren C.J., Bos Ebo, Elewaut D., de Keyser F., Veys E., Joosten I., and Rijnders A.W.M., (1997). Selection of self-reactive peptides within human aggrecan by use of HLA-DRB1*0401 peptide binding motif. J. Autoimmun., **10**: 569-578.

Born T.L., Thomassen E., Bird T.A., and Sims J.E., (1998). Clonning of a novel receptor subunit, AcPL, required for Interleukin-18 signalling. J. Biol. Chem., **273**: 29445-29450.

Borrego F., Robertson M.J., Ritz J., Pena J., and Solana R., (1999). CD69 is a stimulatory receptor for natural killer cell and its cytotoxic effect is blocked by CD69 inhibitory receptors. Immunol., **97**:159-165.

Breedveld F.C., (1999). Future trends in the treatment of rheumatoid arthritis: cytokine targets. Rheumatol., **38**: 11-13.

Brennan F.M., Chantry D., Jackson A., Maini R. and Feldmann M. (1989). Inhibitory effect of TNF-α antibodies on synovial cell and interleukin–1 production in rheumatoid arthritis. Lancet, **29**: 244-247.

Brennan F.M., and Foey A.D., (2002). Cytokine regulation in RA synovial tissue: role of T cell/macrophage contact-dependent interactions. Arthritis Res., **4**: S177-S182.

Brennan P., and O'Neil L.A.J., (1995). Effects of oxidants and antioxidants on nuclear factor κB activation in three different cell lines: evidence against a universal hypothesis involving oxygen radicals. Biochimica et Biophsica Acta, **1260**: 167-175.

Bresnihan B., Maria J., Alvaro-Gracia, Cobby M., Doherty M., Domltjan Z., Emery P., Nuki G., Pavelka K., Rau R., Rozman B., Watt I., Williams B., Aitchison R., McCabe D., and Musikic P., (1998). Treatment of Rheumatoid arthritis with Recombinant human interleukin-1 receptor antagonist. Arthritis Rheum, **41**: 2196-2204

Bresnihan B., (2001). The safety and efficacy of interleukin-1 receptor antagonist in the treatment of rheumatoid arthritis. Semin. Arthritis Rheum., **30**: 17-20.

Brun J.G., Madland T.M., Vedeler C.A., (2002). Immunoglobulin G fc-receptors (FcgammaR) IIA, IIIA, and IIIB polymorphisms related to disease severity in rheumatoid arthritis. J. Rheumatol., 29:1135-1140.

Buras J.A., Reenstra W.R., and Fenton M.J., (1995). NFβA, a factor required for maximal interleukine-1β gene expression is identical to the ets family membrane PU.1. Evidence for structural alteration following LPS activation. Mol. Immunol., **32**: 551-554.

Burger D., and Dayer J-M., (1995). Inhibitory cytokines and cytokine inhibitors. Neurol., **45**: S39-S43.

Burger D., and Dayer J-M. (2002a). The role of human T-lymphocyte-monocyte contact in inflammation and tissue destruction. Arthritis Res., **4**: S169-S176.

Burger D., and Dayer J-M. (2002b). Cytokine, acute-Phase proteins and hormones. IL-1 and TNF-α production in contact-mediated activation of monocytes by T lymphocytes. Ann. N.Y. Acad. Sci., **966**: 464-473.

Burmester G.R., Stuhlmuller B., Keyszer G., and Kinne R.W., (1997). Mononuclear phagocytes and rheumatoid synovitis-mastermind or workhorse in arthritis? Arthritis Rheum., **40**: 5-18.

Buscher D., Hipskind R.A., Krautwald S., Reimann T., and Baccarini M., (1995) Ras-dependent and ras independent pathway target the mitogen-activated protein kinases network in macrophages. Mol. Cell. Biol., **15**:466-475.

Campion G., Lebsack M., Lookabauch J., Gordon G., Catalona M., and the IL-1ra arthritis study group. (1996). Dose-range and dose-frequency study of recombinant human interleukin-1 receptor antagonist in patients with rheumatoid arthritis. Arthritis Rheum., **39**: 1092-1101.

Cantrell D. A., (2002). Phosphoinositide 3-kinase signalling pathways. J. Cell Sci., **114**: 1439-1445.

Carroll R.G., Riley J.L., Levine B.L., Feng Y., Kaushal S., Ritchey D.W., Bernstein W., Weislow O.S., Brown C.R., Berger E.A., June C.H., and St Louis D.C. (1997). Differentail regulation of HIV fusion co-factor expression by CD28 Co-stimulation of $CD4^+$ T cells. Science, **276**: 273-276.

Carruthers, D.M., Naylor, W.G., Allen, M.E., Kitas, G.D., Bacon, P.A, Young, S.P. (1996). Characterization of altered calcium signalling in the T lymphocytes from patients with rheumatoid arthritis (RA). Clin. Exp. Immunol., **105**: 291-296.

Carruthers D.M., Arrol H.P., Bacon P.A, and Young S.P., (2000). Dysregulated intracellular Ca^{2+} strores and Ca^{2+} signalling in synovial fluid T lymphocytes from patients with chronic inflammatory arthritis. Arthritis Rheum., **43**:1-9.

Carson D.A., Pasquali J-L, and Tsoukas C.D, (1981). Physiology and pathology of rheumatoid factor. Springer Semin. Immunopathol., **4**: 161-179.

Castilho R.F., Caravalho-Alves P.C., Vercesi A.E., Ferreira S.T., (1996). Oxidative damage to sarcoplasmic reticulum Ca $^{(2+)}$–pump induced by Fe^{2+}/H_2O_2/ascorbate is not mediated by lipid peroxidation or thiol oxidation and leads to protein fragmentation. Mol. Cell Biochem. **159**: 105-114.

Cemerski S., van Meerwijk J.P.M., and Romagnoli P., (2003). Oxidative-stress-induced T lymphocyte hyporesponsiveness is caused by structural modification rather than proteasomal degradation of crucial TCR signalling molecules. Eur. J. Immunol., **33**: 2178-2185.

Cerretti D., Kozlosky C., Mosley B., Nelson N., Ness K., Greenstreet T., March C., Kronheim S., Druck T., Cannizzaro L., Huebner K., Black R., (1992). Molecular cloning of the interleukin-1β converting enzymes. Science, **256**: 97-100.

Chabaud M., Durand J.M., Buchs N. Fossiez F. Page G., Frappart L. and Miossec P. (1999). Human interleukin-17: A cell derived proinflammatory cytokine produced by the rheumatoid synovium. Arthritis Rheum., **42**: 963-970.

Chakravarti B., and Abraham G.N., (2002). Effect of age and oxidative stress on tyrosine phosphorylation of ZAP-70. Mech. Age. Develop., **123**: 297-311.

Chandel N.S., Trzyna W.C., McClintock D.S., and Schumacker P.T., (2000). Role of oxidants in NF-κB activation and TNF-α gene transcription induced by hypoxia and endotoxin. J. Immunol., **165**: 1013-1021.

Chandhri G., Hunt N.H., Clark I.A, and Cereding R., (1988). Antioxidants inhibit proliferation and cell-surface expression of receptors for interleukin-2 and transferring in lymphocytes-T stimulated with phorbol-myristrate acetate and ionomycin. Cell. Immunol., **115**: 204-213.

Chang D-M., Weinblatt M.E., and Schur P.H., (1992). The effect of methotrexate on interleukin-1 in patients with rheumatoid arthritis. J. Rheumatol., **19**: 1678-1682.

Chikanza I.C., Kingsley G. and Panayi G.S., (1995). Peripheral-blood and synovial fluid monocytes expression of interleukin-1 alpha and beta during active rheumatoid arthritis. J. Rheumatol., **22**: 600-606.

Chin J., and Kostura M.J., (1993). Dissociation of IL-1β synthesis and secretion in human blood monocytes stimulated with bacterial cell wall products. J. Immunol., **151**: 5574-5585.

Chizzolini C., Chicheportiche R., Burger D. and Dayer J-M., (1997). Human Th1 cells preferentially induce interleukin-1 (IL-1)-β while Th2 induce IL-1 receptor antagonist production upon cell/cell contact with monocytes. Eur. J. Immunol., **27**: 171-177.

Chizzonite R., Truitt T., Kilian P.L., Stern A.S., Nunes P., Parker K.P., Kaffka K.L., Chua A.O., Lugg D.K., and Gubler U., (1989). Two high affinity interleukin-1 receptors represent seprate gene-products. Proc.Natl. Acad. Sci., USA, **86**: 8029-8033

Choi Y.W., Herman A., Digiusto D., Wade T., Marrack P. and Kappler J., (1990). Residues of the variable region of the T cell-receptor beta-chain that interact with staphylococcus-aureus toxin superantigens. Nature, **346**: 471-473

Chow J. C., Young D. W., Golenbock D.T., Chris W.J., and Gusovsky F., (1999). Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction, J. Biol. Chem., **274**: 10689-10692.

Coan, C., Ji J., Hideg K., Mehlhorn R., J, (1992). Protein sulfhdryls are protected from irreversible oxidation by conversion to mixed disulfides. Arch. Biochem. Biophys, **295**: 369-378.

Coclet-Ninin J., Dayer J-M., and Burger D., (1997). Interferon-beta not only inhibits interleukin-1beta and tumour necrosis factor-alpha but stimulates interleukin-1 receptor antagonist production in human peripheral blood mononuclear cells. Eur. Cytokine Net., **8**: 345-349.

Coffman R.L, Lebman D.A. and Sharader B., (1989). Transforming growth factor-beta specifically enhances IgA production by LPS-stimulated murine lymphocytes B. J. Exp. Med., **170**: 1039-1044.

Collotta F., Re F., Muzio M., Bertini R., Polentarutti N., Sironi M., Giri J.G., Dower S.K., Sims J.E., and Mantovani A., (1993). Interleukin-1 type-II receptor a decoy target for IL-1 that is regulated by IL-4. Science, **261**: 472-475.

Collotta F., Re F., Muzio M., Polentarutti N., Minty A., Caput D., Ferrara P., and Mantovani A., (1994). Interleukin-13 induces expression and release of interleukin-1 decoy receptor in human polymorphonuclear cells. J. Biol. Chem., **269**: 12403-12406.

Constant S.L., and Bottomly K., (1997). Induction of Th1 and Th2 $CD4^+$ T cell responses: the alternative approaches. Ann. Rev. Immunol., **15**: 297-322.

Cook J. A., Iype S. N., and Mitchell J.B. (1991). Differential specificity of monochlorobimane for isozyme of human and rodent glutathione S-transferases. Cancer Res. **51**: 1606-1612.

Cope A.P., (2002). Studies of T cell activation in chronic inflammation. Arthritis Res., **4**: S197-S211.

Correia J.S., and Ulevitch R., (2002). MD-2 and TLR-4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. J. Biol. Chem., **277**:1845-1854.

Corrigall VM, Panayi G.S., (2002). Autoantigens and immune pathways in rheumatoid arthritis. Crit. Rev. Immunol., **22**: 281-293.

Cottrez F., and Groux H., (2004). Specialization in tolerance: innate $CD4^+CD25^+$ versus acquired TR1 and TH3 regulatory T cells. Transplantation, **77**: S12-S15.

Crofford, L.J., Tan B., Mccarthy, C.J., and Hla, T. (1997). Involvement of nuclear factor κB in the regulation of cyclooxygenase-2 expression by interleukin-1 in rheumatoid synoviocytes. Arthritis Rheum., **40**: 226-236.

Cunnane G., Madigan A., Murphy E., FitzGerald O., Bresniham B., (2001). The effect of treatment with interleukin-1 receptor antagonist on the inflamed synovial membrane in rheumatoid arthritis. Rheum., **40**: 62-69.

Czernieki B.J., Carter C., Rivoltini L., Koski G.K., Kim H.I., Weng D.E., Roros J.G., Hijazi Y.M., Xu S.W., Rosenberg S.A., and Cohen P.A., (1997). Calcium ionophore-treated periheral blood monocytes and dendritics cells rapidly display characteristics of activated dendritic cells. J. Immunol., **159**: 3823-3837.

Dayer J-M., & Fenner H. (1992). The role of cytokine and their inhibitors in arthritis. Baillier's Clin. Rheumatol., **6**: 486-516.

Dayer J-M., & Bresnihan B., (2002). Targeting interleukin-1 in the treatment of rheumatoid arthritis. Arthitis Rheum., **46**: 574-578.

Dayer J-M, (2003). How T-lymphocytes are activated and become activators by cell-cell contact. Eur. Respir. J., **22**: 10s-15s.

De Benedetti F., Pignatti P., Massa M., Sartirana P., Ravelli A., and Martini A., (1995). Circulating levels of interleukin-1 beta and of interleukin-1 receptor antagonist in systemic juvenile chronic arthritis. Clin. Exp. Rheumatol., **13**: 779-784.

De Maria R., Cifone M.G., Trotta R., Rippo M.R., Festuccia C. Santoni A., and Testi R. (1994). Triggering of human monocytes activation through CD69, a member of the natural killer cells gene complex family of signal transducing receptors. J. Exp. Med., **180**: 1999-2004.

De Stahl T.D., Andren M., Martinsson P., Verbeek J.S., and Kleinau S., (2002). Expression of Fcγ RIII is required for development of collagen-induced arthritis. Eur. J. Immunol., **32**: 2915-2922.

Dean N.M., Mackay R., Condon T.P, and Bennett C.F., (1994). Inhibition of protein-kinase C-alpha expression in human A549 cells by antisense oligonucleotide inhibits induction of intracellular-adhesion molecule-1 (ICAM-1) messnger-RNA by phorbol esters. J. Biol. Chem. **269**: 16416-16424.

Deon D., Ahmed S., Tai K., Scaletta N., Herrero C., Lee I-H., Krause A., and Ivashkiv L. B, (2001). Cross Talk between IL-1 and IL-6 Signalling pathways in Rheumatoid arthritis synovial fibroblasts. J. Immunol., **167**: 5395-5403.

Dinarello C.A., (1996). Biological basis for interleukin-1 in disease. Blood, **87**: 2095-2147.

Doan T.N., Gentry D.L., Taylor A.A., Elliott S.J., (1994). Hydrogen peroxide activates agonsit-sensitive Ca^{2+} flux pathways in canine venous endothelial cells. Biochem J., **297**: 209-215.

Dobashi K., Aihara M., Araki T., Shimizu Y., Utsugi M., Iizuka K., Murata Y., Hamura J., Nakazawa T., and Mori M., (2001). Regulation of LPS induced IL-12 production by IFN-γ and IL-4 through intracellular glutathione status in human macrophages. Clin. Exp. Immunol., **124**: 290-296.

Dolhain R.J.E., Heiden A.N., Haar N., Breedveld F. and Miltenburg A.M., (1996). Shift toward T lymphocytes with a T helper 1 cytokine-secretion profile in the joint of patient with rheumatoid arthritis. Arthritis Rheum, **39**: 1961-1969.

Dolhain R.J.E., Tak P.P., Dijkmans B.A.C., Kuiper P.D.E., Breedveld F.C. and Miltenburg A.M., (1998). Methotrexate reduces inflammatory cell numbers, expression of monokines and of adhesion molecules in synovial tissue of patients with rheumatoid arthritis. Br. J. Rheumatol., **37**: 502-508.

Duevadas S., Zaritskaya L., Rhee S.G., Oberley and Williams M.S., (2002). Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: Selective regulation of mitogen-activated protein kinase activation and Fas ligand expression. J. Exp. Med., **195**: 59-70.

El Gabalawy H., Gallatin M., Vazeux R., Peterman G., and Wilkins J., (1994). Expression of ICAM-R (ICAM-3), a novel counter-receptor for LFA-1, in rheumatoid and nonrheumatoid synovium-comparison with other adhesion molecules. Arthritis Rheum., **37**: 846-854.

Elliott S.J., Meszaros J.G., Schilling W.P., (1992). Effect of oxidative stress on calcium signalling in vascular endothelial cells. Free Rad. Biol. Med., **13**: 635-650.

Ermak G., and Davies K., (2001). Calcium and oxidative stress: from cell signalling to cell death. Mol. Immunol., **38**: 713-721.

Fairburn K., Stevens C.R., Winyard P.G., Kus M., Ward R.J., Cunningham J., Zaidi M., and Blake D.R., (1993). Oxidative stress and its control: A pathogenetic role in inflammatory joint disease. Biochem. Soc. Trans., **21**: 371-375.

Fehniger T.A., and Caligiuri M.A.,(2001). Interleukin-15: Biology and relevance to human diseases. Blood, **97**:14-32.

Feldmann M., Brennan F.M., and Maini N., (1996). The role of cytokines in rheumatoid arthritis. Ann. Rev. Immunol, **14**: 397-440.

Feldmann M. and Maini R., (1999). The role of cytokines in the pathogenesis of rheumatoid arthritis. Rheumatol., **38**: 3-7.

Fernandes J., Martel-Pettletier J., and Pelletier J.P., (2002). The role of cytokines in osteoarthritis pathophysiology. Biorheology, **39**: 237-246.

Finkelman F.D., Wynn T.A., Donaldson D.D. and Urban Jr J., (1999). The role of IL-13 in helminth-induced inflammation and protective immunity against nematode infections. Curr. Opin. Immunol., **11**: 420-426.

Firestein G.S., Roeder W.D., Laxer J.A., Townsend K.S., Weaver C.T., Hom J.T, Linton J., Torbett B.E., and Glasebrook A.L., (1989). A new murine $CD4^+$ T cell subset with an unrestricted cytokine profile. J. Immunol., **143**: 518-525.

Firestein G.S., Alvaro-Garcia and Maki R., (1990). Quantitative analysis of cytokine gene expression in rheumatoid arthritis. J. Immunol., **144**: 3347-3353.

Firestein G., Boyle D., Yu C., Paine M., Whisenand T., Zvaifler N., Arend W., (1994). Synovial interleukin-1 balance in rheumatoid arthritis. Arthritis Rheum., **37**:644-652.

Forslind K., Svensson B., Sevenson M., Bendtzen R., (2001). Anti-IL-1α autoantibodies in early rheumatoid arthritis. Scand. J. Rheumtol., **30**: 167-168.

Foussat A., Cottrez F., Brun V., Fournier N., Breittmayer J-P, and Groux H., (2003). A comparative study between T regulatory type 1 and $CD4^+$ $CD25^+$ T cells in the control of inflammation. J. Immunol., **171**: 5018-5026.

Fries J.F., (2000). Current treatment paradigms in rheumatoid arthritis. Rheumatol., **39**: 30-35.

Ghayur T., Banerjee S., Hugunin M., Butler D., Herzog L., Carter A., Quintal L., Sekurt L., Talanian R., Paskind M., Wong W., Kamen R., Tracey D., Allen H., (1997). Caspase-1 processes IFN-γ inducing factor and regulates LPS-induced INF-γ production. Nature, **386**: 619-623.

Ghezzi P., Dinarello C.A., Bianchi M., Rosandich M., Repine J., White C., (1991). Hypoxia increases production of interleukin-1 and tumour necrosis factor by human mononuclear cells. Cytokine, **3**: 189-194

Ghosh S., May M.J., and Kopp E.P., (1998). NF-κB and Rel proteins: Evolutionarily conserved mediators of immune responses. Ann. Rev. Biomed. Sci., **16**: 225-260.

Giambartolomei G.H., Dennis V.A., Lasater B.L., and Philip M.T., (1999). Induction of pro and anti-inflammatory cytokines by Borrelia Burgdorferi lipoproteins in monocytes is mediated by CD14. Infec. Immun., **67**:140-147.

Ginn-Pease M., and Whisler R.L., (1998). Redox signals and NF-κB activation in T cells. Free Rad. Bio. Med., **25**: 346-361.

Glant T.T., Mikecz K., Arzoumanian A., and Poole A.R., (1987). Proteoglycan-induced arthritis in balb/c mice-clinical-features and histopathology. Arthritis Rheum., **30**: 201-212.

Goldstone S.D., and Hunt N.H., (1997). Redox regulation of the mitogen-activated protein kinase pathway during lymphocyte activation. Biochimica et Biophysica acta, **1355**: 353-360

Gomez-Reino J.J, Pablos J.L., Carreira P.E., Santiago B., Serrano L., Vicario J.L, Balsa A., Figueroa M. and De Juan M.D., (1999). Association of rheumatoid arthritis with a functional chemokine receptor, CCR5. Arthritis Rheum., **42**: 989-992.

Gopalakrishna R., and Jaken S., (2000). Protein kinase C signalling and oxidative stress. Free Rad. Bio. Med., **28**: 1349-1361.

Goronzy J, Zeth A., Weyand C., (1998). T cells receptor repertoire in rheumatoid arthritis. Int. Rev. Immunol., **17**: 339-363.

Greenfeder S.A, Nunes P, Kwee L., Labow M., Chizzonite P.A. and Ju G., (1995). Molecular cloning and characterization of a 2nd subunit of the interleukine-1 receptor complex. J. Biol. Chem., **270**:13757-13765.

Gregersen P.K, Silver J. and Winchster R.J., (1987). The shared epitope hypothesis: an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. Arthritis Rheum., **30**: 1205-1213.

Grewal I.S, and Flavell R. A. (1998). CD40 and CD154 in cell-mediated immunity. Ann. Rev. Immunol., **16**: 111-135.

Griffiths E.J., Ocampo C.J., Savage J.S., Rutter G.M., Hansford R.G., Stern M.D., and Silverman H.S., (1998). Mitochondrial calcium transporting pathways during hypoxia and reoxygenation in single rat cardiomyocytes. Cardiovasc. Res., **39**: 423-433.

Gringhuis S.I., Leow A., Papendrecht-Van der Voot, Remans P.H.J, Breedveld F.C. and Verweij C.L (2000). Displacement of Linker for activation of cells from the plasma membrane due to redox balance alterations results in hyporesponsiveness of synovial fluid T cells in rheumatoid arthritis. J. Immunol., **164**: 2170-2179.

Gringhuis S.I., Ellen A.M., Papendrecht-Van der Voot, Leow A, Nivine Levarht E.W., Ferdinand C., Breedveld F.C, and Verweij C.L (2002). Effect of redox alteration on cellular localization of LAT and downstream T-cell receptor signalling pathways. Mol. Cell. Biol., 22:400-411.

Groux H., and Powrie F., (1999). Regulatory T cells and inflammatory bowel disease. Immunol. Today, **20**: 442-445.

Groux H., (2001). An overview of regulatory T cells. Microbes and Infec., **3**: 883-889.

Grover A.K., Samson S.E., and Fomin V.P., (1992). Peroxide inactivates calcium pumps in pig coronary artery. Am. J. Physiol., **263**: H537-H543.

Grube B.J., Cochane C.G., Ye R.D., Green C.E., Mcphail M.E., Ulevitch R.J., and Tobias P.S., (1994). Lipopolysaccharide-binding protein expression in primary human hepatocytes and Hep-γ 2 hepatoma-cells. J. Biol. Chem., **269**: 8477-8482.

Guinan E.C., Gribben J.G., Boussiotis V.A., Freeman G.J., and Nadler L.M., (1994). Pivotal role of the B7-CD28 pathway in transplantation tolerance and tumor-immunity. Blood, **84**: 3261-3282

Haddad J.J., (2001a). Glutathione depletion is associated with augmenting a proinflammatory signal: evidence for an antioxidant/prooxidant mechanism regulating cytokines in the alveolar epithelium. Cytokine Cell Mol. Ther., **6**: 177-187.

Haddad J.J., (2001b). L-buthionine-(S,R)-sulfoximine, an irreversible inhibitor of gamma-glutamylcysteine synthetase, augments LPS-mediated pro-inflammatory cytokines biosyenthesis: evidence for the implication of an IkappaB-alpha/NF-kappa B insensitive pathway. Eur. cytokine. Netw., **12**: 614-624.

Haddad J.J., (2002a). The involvement of L-γ-glutamyl-l-cysteinyl-glycine (glutathione/GSH) in the mechanism of redox signalling mediating $MAPK^{p38}$-dependent regulation of pro-inflammatory cytokine production. Biochem. Pharmacol., **63**: 305-320.

Haddad J.J., (2002b). Antioxidant and prooxidant mechanisms in the regulation of redox (y)-sensitive transcription factors. Cell. Signal., **14**: 879-897.

Haddad J.J., (2002c). Redox regulation of proinflammatory cytokines and IkB-α / NF-κB nuclear translocation and activation. Biochem. Biophys. Res.Commun., **296**: 847-856

Haddad J., and Fahlman C.S., (2002). Redox-and oxidant-mediated regulation of interleukin-10: an anti-inflammatory, antioxidant cytokine? Biochem. Biophys. Res. Commun., **297**: 163-176.

Haddad J, and Land S.C., (2002). Redox signalling-mediated regulation of lipopolysaccharide-induced proinflammatory cytokine biosynthesis in alveolar epithelial cells. Antioxid. Redox Signal., **4**: 179-193.

Hailman E., Vasselon M., Kelley L.A., Busse C.T., Mickey H.S., Lichenstein P.A., Detmers P.A., and Wright S.D., (1996). Stimulation of macrophages and neutrophils by complexes of lipopolysaccharide and soluble CD14. J. Immunol., **156**: 4384-4390.

Hale K., Trollinger D., Rinhanek, and Manthey M. (1999). Differential expression and activation of p38 mitogen- activated protein kinase α, β, γ and δ in inflammatory cell lineages. J. Immunol., **162**: 4246-4252.

Halliwell B., Hoult J.R., and Blake D.R., (1988). Oxidants, inflammation, and anti-inflammatory drugs. FASEB J., **2**: 2867-2873.

Han Z., Boyle D., Chang L., Bennet B., Karin M., Yang L., Manning A., and Firestein G.S., (2001). c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. J. Clin. Invest., **108**: 73-81.

Harada S., Yamamura M., Okamoto H., Morita Y., Kawashima M., Aita T., Makino H., (1999). Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum., **42**:1508-1516.

Harris E.D., (1990). Mechanisms of disease-rheumatoid arthritis-pathophysiology and implication for therapy. New England J. Med., **322**: 1277-1289.

Harris S.G., Padilla J., Koumas L., Ray D., and Phipps R., (2002). Prostaglandins as modulators of immunity. Trends Immunol., **23**: 144-150

Hart P.H., Vitti G.F., Burgess, D.R., Whitty G.A., Piccoli D.S and Hamilton J.A., (1989). Potential anti-inflammatory effect of interleukine-4 suppression of human monocytes tumor necrosis factor-alpha, interleukin-1 and prostaglandin E_2. Proc. Natl. Acad., Sci., USA, **86**: 3803-3807.

Hashimoto S., Gon Y., Matsumoto K., Takeshita I., Machino T., and Horie T., (2001). Intracellular glutathione regulates tumour necrosis factor-α induced p38 MAP kinase activation and RANTES production. Clin. Exp. Allergy, **31**: 144-151.

Hashimoto S., Gon Y., Matsumoto K., Takeshita I., and Horie T., (2001). N-acetylcysteine attenuates TNF-α -induced p38 MAP kinase-mediated IL-8 production by human pulmonary vascular endothelial cells. Br. J. Pharmacol., **132**: 270-276.

Hayes J. D., and McLellan I., (1999). Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Rad. Res., **31**: 273-300.

Haziot A., Lin X.Y., Zhang F., and Goyert S.M., (1998). Cutting Edge: The induction of acute phase protein by lipopolysaccharide uses a novel pathway that is CD14-independent. J.Immunol., **160**: 2570-2572.

Hermann A., Kappler J.W., Marrack P., and Pullen A.M, (1991). Superantigens: Mechanism of T cell stimulation and role in immune responses. Annu. Rev. Immunol, **9**: 745-772.

Hermann G., Harper L., Schaible T., (1999). Summary of clinical trials in rheumatoid arthritis using infliximab, an anti-TNF-α treatment. Ann. Rheum. Dis., **58**: 161-164.

Hermann J., Hall M.A., Maini R., Feldmann M. and Brennan F. M. (1998). Important immunoregulatory role of interleukin–11 in the inflammatory process in rheumatoid arthritis. Arthritis Rheum., **41**: 1388-1397.

Hilkens C.M.U., Kalniski P., de Boer M., and Kapsenberg M.L., (1997). Human dendritic cells require exogeneous interleukin-12 inducing factors to direct the development of naïve T helper cells toward the Th1 phenotype. Blood, **90**: 1920-1926.

Hmama Z., Knutson K., Herrera-Velit P., Nandan D., and Reiner N.E., (1999). Monocytes adherence induced by lipopolysaccharide involves CD14, LFA-1, and cytohesin-1. J. Biol. Chem., **274**: 1050-1057.

Hogquist K.A., Nett M.A., Unanue E.R., and Chaplin D.D., (1991). Interleukin-1 is processed and released during apoptosis. Proc. Natl. Acad. Sci., USA, **88**: 8485-8489.

Holoshitz J., Matitiau A., and Cohen I.R., (1984). Arthritis induced in rats by cloned T lymphocyes responsive to mycobacteria but not to collagen type II. J. Clin. Invest., **73**: 211-216

Honda S., Migita K., Hirai Y., Origuchi T., Yamasaki S., Kamachi M., Shibatomi K., Fukuda T., Kita M., Hida A., Ida H., Aoyagi T., Kawakami A., Kawabe Y., Oizumi K., and Euchi K., (2001). Expression of membrane-type I matrix metalloproteinase in rheumatoid synovial cells. Clin. Exp. Immunol., **126**: 131-136.

Houssein D., Vincent C., Serre G., Scott D., (1996). Anti-keratine antibodies, disease activity and radiological damage in RA. Br. J. Rheumatol. **35** (suppl.2): 14-17.

Hyrich K.L. and Inman R.D., (2001). Infectious agents in chronic rheumatic diseases. Curr. Opin. Rheumatol., **13**: 300-304.

Iho S., Takahashi T., Kura F., Sugiyama H, and Hoshino T., (1986). The effect of 1,25 dihydroxyvitamin-D3 on *in vitro* immunoglobulin production in human B cells. J. Immunol., **136**: 4427-4431.

Isler P., Vey E., Zhang J-H., and Dayer J-M. (1993). Cell surface gylcoproteins expressed on activated human T cells induce production of interleukin-1 beta by monocytes cells: possible role of CD69. Eur. Cytokine. Netw., **4**: 15-23

Jablonska E., Izycka A., Jablonska J., Wawrusiewicz N., Piecuch J., (2001). Role of IL-18 in the secretion of IL-1beta, sIL-1RII, and IL-1ra by human neutrophils. Immunol. Invest., **30**: 221-229.

Janssen-Heininger Y.M.W., Macara I., and Mossman B.T., (1999). Cooperativity between oxidants and tumor necrosis factor in the activation of nuclear factor (NF)-κB. Am. J. Respir. Cell Mol. Biol., **20**: 942-952.

Jeannin P., Delneste Y., Lecoanet-Henchoz S., Gauchat J-F., Life P., Holmes D., and Bonnefoy J-Y., (1995). Thiols decrease human interleukin (IL) 4 production and IL-4-induced immunolglobulin synthesis. J. Exp. Med., **182**: 1785-1792

Jenkins J.K., Drong R.F., Shuck M.E., Bienkowski M.J., Slightom J.L., Arend W.P., and Smith Jr. M.F., (1997). Intracellular IL-1 receptor antagonist promoter. J. Immunol., **158**: 748-755.

Jiang Q., Akashi S., Miyake K., and Petty H.R., (2000). Cutting Edge: Lipopolysaccharide induces physical proximity between CD14 and Toll-Like Recepors 4 (TLR 4) prior to nuclear translocation of NF-κB. J. Immunol, **165**: 3541-3544.

Jovanovic D.V., Di Battista J.A., Martel-Pelletier J., Jolicoeur F. C., He Y., Zhang M., Mineau F, and Pelletier J-P. (1998). IL-17 stimulates the production and expression of proinflammatory cytokine IL-1β and TNF-α by human macrophages. J. Immunol, **160**: 3513-3521.

June C.H., Bluestone J.A., Nadler L.M., and Thompson C.B., (1994). The B7 and CD28 receptor families. Immunol. Today, **15**: 321-331.

Kamata H., and Hirata H., (1998). Redox regulation of cellular signalling. Cell signal, **11**: 1-14.

Kane L.P., Lin J., and Weiss A., (2002). It's all Rel-ative: NF-κB and CD28 costimulation of T cell activation. Trends in Immunol., **23**: 413-420.

Kashiwamura S., Ueda H., Okamura H., (2002). Roles of interleukin-18 in tissue destruction and compensatory reactions. J. Immuno. Ther, **25**: S4-S11

Katsikis P.D., Chu C.Q., Brennan F.M., Maini RN., Feldmann M., (1994). Immunoregulatory role of interleukin 10 in rheumatoid arthritis. J. Exp. Med., **179**: 1517-1527.

Kavanagh T. J., Martin G. M, Livesey J. and Rabinovitch P. (1988). Direct evidence of intracellular sharing of glutathione via metabolic cooperation. J. Cell. Phys., **137**: 353-359.

Kavanagh T. J., Raghu G., White C.C., Martin G. M., Rabinovitch P., Eaton D. (1994). Enhancement of glutathione content in glutathione synthetase-Deficient fibroblasts from a patient with 5-oxoprolinuria via metabolic cooperation with fibroblasts. Exp. Cell Res. **212**: 69-76

Keffer J., Probert L., Cazlaris H., Geargopoulos S., Kaslaris E., Kioussis D., and Kollias G., (1991). Transgenic mice expressing human tumour necrosis factor: a predictive genetic model of arthritis. EMBO, **10**: 4025-4031.

Kidd P., (2003). Th1/Th2 balance: The hypothesis, its limitations, and implications for health and disease. Altern. Med. Rev., **8**: 223-246.

Kinne R.W., Palombo-Kinne E., and Emmrich F., (1997). T cells in the pathogensis of rheumatoid arthritis Villians or accomplices? Biochimica et Biophysica Acta, **1360**: 109-141.

Kitchens R. L, and Munford R.S. (1998). CD14-dependent internalisation of bacterial lipopolysaccharide (LPS) is strongly influenced by LPS aggregation but not by cellular response to LPS. J. Immunol., **160**: 1920-1928.

Kitchens R. L, Wang P., and Munford R.S. (1998). Bacterial lipopolysaccharide (LPS) can enter monocytes via two CD-14-dependent pathways. J. Immunol, **161**: 5534-5545.

Klimiuk P.A., Yang H. Groronzy J.J., and Weyand C.M., (1999). Production of cytokines and metalloproteinases in rheumatoid synovitis T cell dependent. Clin. Immunol., **90**: 65-78.

Koch A.E., Kunkel S.L., Harlow L.A., Johnson B., Evanoff, H.L., Haines G.F., Burdick M.D., Pope R.M., and Stieter R.M., (1992). Enhanced production of monocytes chemoattractant protein-1 in rheumatoid arthritis. J. Clin. Invest., **90**: 772-779.

Kokura S., Wolf R., Yoshikawa T., Granger D.N., Aw T.Y., (1999). Molecular mechanisms of neutrophil-endothelial cell adhesion induced by redox imbalance. Circ. Res., **84**: 516-524.

Kono K., SalazarOnfray F., Petersson M., Hansson J., Masucci G., Wasserman K., Nakazawa T., Anderson P., and Kiessling R., (1996). Hydrogen peroxide secreted by tumor-derived macrophages down-modulates signal-transducing zeta molecules and inhibits tumor-specific T cell and natural killer cell-mediated cytotoxicity. Eur. J. Immunol., **26**: 1308-1313.

Kostura M. J., Tocci M.J, Limjuco G., Chin J., Cameron P., Hillman A.G., Chartrain N.A., and Schmidt J. A. (1989). Identification of a monocytes specific pre-interleukin-1 β convertase activity. Proc. Natl. Acad. Sci., **86**: 5227-5231.

Kramer R.A., Greene K., Ahmad S., and Vistica DT., (1987). Chemosensitization of L-phenylalanine mustard by the thiol-medulating agent buthionine sulfoximine. Cancer Res., **47**: 1593-1597.

Krishnan B.R., (1999). Interleukin-1 receptor antagonist therapy for rheumatoid arthritis. Curr. Opin. Mol. Ther., **1**: 454-457.

Kumar S., McDonnell P., Lehr R., Tierney L., Tzimas M., Grisworld D., Capper E., Tal-Singer R., Wells G., Doyle M.L., (2000). Identification and initial characterization of four novel members of the interleukin-1 family. J. Biol. Chem., **275**: 10308-10314.

Kunkel S. L., Lukacs N., Kasama T., Strieter R.M. (1996). The role of chemokines in inflammatory joint disease. J. Leuk. Biol., **58**: 6-12.
Kupfer A., (2000). Breaking up receptor alliances: the parting of CD3 and CD4. Nature Med., **6**: 1097-1098.

Kurosaka K., Watanabe N., and Kobayashi Y., (1998). Production of proinflammtory cytokines by phorbol myristate acetate treated THP-1 cells and monocytes-derived macrophages after phagocytosis of apoptotic CTL-2 cells. J. Immunol., **161**: 6245-6249.

Lacraz S., Isler P., Vey E., Welgus H., and Dayer J-M. (1994). Direct contact between T lymphocytes and monocytes is a major of induction of Metalloproteinase Expression. J. Biol. Chem., **269**: 22027-22033

LaDuca JR., Gaspari AA., (2001). Targeting tumor necrosis factor alpha. New drug used to modulate inflammatory diseases. Dermatol Clin., **19**: 617-635.

Lafyatis R., Thompson N.L., Remmers E.F., Flanders K.C., Roche N.S., Kim S.J., Case J.P., Sporn M.B., Roberts A.B., and Wilder R.L., (1989). Transforming growth factor-beta production by synovial tissue from rheumatoid patients and streptococcal cell-wall arthritic rats-studies on secretion by synovial fibroblast-like cells and immunohistologic localization. J. Immunol., **143**: 1142-1148.

Laliberte R.E., Eggler J., and Gabel C.A., (1999). ATP Treatment of Human monocytes promotes caspase-1 maturation and externalization. J. Biol. Chem., **274**: 36944-36951.

Landman R., Link S., Sansano S., Rajacic Z., and Zimmerli W., (1998). Soluble CD14 activates monocytic cells independently of lipopolysaccharide. Infec. Immun., **66**: 2264-2271.

Lee S.H., Soyoola E., Chanmugan P., Hart S., Sun W.Q., Zhong H., Liou S., Simmons D. and Hwang D., (1992). Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. J. Biol. Chem., **267**: 25934-25938.

Lemire J.M., Adams J.S., Kermaniarab V., Bakke A.C., Sakai R., and Jordan S.C., (1985). 1,25-Dihydroxyvitamin-D3 suppresses human T helper inducer lymphocytes activity in vitro. J. Immunol., **134**: 3032-3035.

Letterio J.J.,& Roberts A.B., (1998). Regulation of immune responses by TGF-β. Ann. Rev. Immunol., **16**: 137-161.

Liang C-M., Lee N., Cattell D., and Liang S-M., (1989). Glutathione regulates interleukin-2 activity on cytotoxic T cells. J. Biol. Chem., **264**: 13519-13523.

Lien E., Means T.K., Heine H., Yoshimura A., Kusumoto S., Fukase K., Fenton M.J., Oikawa M., Qureshi N., Monks B., Finberg R.W., Ingalls R.R., and Golenbock D., (2000). Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J. Clin. Invest., **105**: 497-504.

Liote F., BovalBoizard B., Weill D., Kuntz D., and Wautier J.L., (1996). Blood monocytes activation in rheumatoid arthritis: Increased monocyte adhesiveness, integrin expression expression, and cytokine release. Clin. Exp. Immunol., **106**: 13-19.

Liu M., Kohsaka H., Sakurai H., Azuma M., Okumura K., Saito I and Miyasaka N. (1996) The presence of costimulatory molecule CD86 and CD28 in rheumatoid arthritis synovium. Arthritis Rheum, **39**:110-114.

Liu C-M, and Hermann T.E., (1978). Characterization of ionomycin as a calcium ionophore. J. Biol. Chem., **253**: 5892-5894.

Lochner M., Wagner H., Classen M., Forster I., (2002). Generation of neuralizing mouse anti-mouse IL-18 antibodies for inhibition of inflammatory responses in vivo. J. Immunol. Meth., **259**: 149-157.

Loetscher H., Gentz R., Zulauf M., Lustig A., Tabochi H., Schlaeger, E.J., Brockhaus M., Gallati H., Manneberg M. and Lesslauer W., (1991). Recombinant 55-kDa tumor-necrosis factor (TNF) receptor-stoichiometry of binding to TNF-alpha and TNF-beta and inhibition of TNF activity. J. Bio. Chem., **266**: 18324-18329.

Lohmander L.S., Hoerrner L.A., Dahlberg L., Roos H., Bjornsson S., and Lark M.W., (1993). Stromelysin, tissue inhibitor of metalloproteinases and proteoglycan fragments in human knee-joint fluid after injury. J. Rheumatol., **20**: 1362-1368.

Lynch D.R., and Dawson T.M., (1994). Secondary mechanisms in neural trauma. Curr. Opin. Neurol., **7**: 510-516.

Maini R., Breedveld F., Kalden J., Smolen J., Davis D., Macfarlane J., Antoni C., Leeb B., Elliott M., Woody J., Schaible T., and Feldmann M., (1998). Therapeutic efficacy of multiple intravenous infusions of anti-tumor necrosis factor α monoclonal antibody combined with low-dose weekly methotrexate in rheumatoid arthritis. Arthritis Rheum., **41**: 1552-1563.

Malik J., Greenfield B.W., Wahl A., and Kiener P. A. (1996). Activation of human monocytes through CD40 induces matrix metalloproteinases. J. Immunol., **156**: 3952-3960.

Malyak M., Guthridge J., M., Hance K., Dower S., Freed J., and Arend W., (1998). Characterization of the low molecular weight isoform of IL-1 receptor antagonist. J. Immunol., **161**: 1997-2003.

Manicourt D-H., Fujimoto N., Ken'ichi O., and Thonar E.J-M., (1995). Levels of circulating collagenase, stromelysin-1, and tissue inhibitor of matrix metalloproteinase1 in patients with rheumatoid arthritis. Arthritis Rheum., **38**: 1031-1039.

Manie S., Kubar J., Limouse M., Ferrua B., Ticchioni M., Breitmayer P., Peyron J-F., Schaffar L., and Rossi B. (1993). CD3-stimulated Jurkat T cells mediate IL-1β production in monocytes THP-1 cells. Role of LFA-1 molecule and participation of CD69 T cells antigen. Eur. Cytokine. Netw, **4**: 7-13.

Mapp P.I., Grootveld, and Blake D.R., (1995). Hypoxia, oxidative stress and rheumatoid arthritis. Br. Med. Bull., **51**: 419-436.

Masferrer J.L. Zweifel B.S., Manning P.T., Hauser S.D., Leahy K.M., Smith W.G., Isakson P. C., and Seibert K., (1994). Selective inhibition of inducible cyclooxygenase 2 in vivo is anti-inflammatory and nonnulcerogenic. Proc. Natl. Acad. Sci., **91**: 3228-3232.

Mattern T., Thanhauser A., Reiling N.,. Toellner K.M., Duchrow M., Kusumoto S., Rietschel E.T., Ernst M., Brade H., Flad H.D., (1994). Endotoxin and lipid A stimulate proliferation of human T cells in the presence of autologous monocytes. J. Immunol., **153**: 2996-3004.

Mattern T., Flad H.D., Brade L., Rietschel E. T., Ulmer A.J (1998). Stimulation of human T lymphocytes by LPS is MHC unrestricted, but dependent on B7 interaction. J. Immunol., **160**: 3412-3418.

Mattern T., Girroleit G., Flad H.D., Rietschel E. T., Ulmer A.J (1999). Haematopoietic stem cells exert accessory function in lipopolysaccharide-induced T cell stimulation and CD80 expression on monocytes. J. Exp. Med., **189**: 693-700.

Maurice M.M., Nakamura H., Vander Voort E.A.M, Van Vliet A.I., Staal F.J.T., Tak P.P., Breedveld F.C., and Verweij C.L., (1997). Evidence for the role of an altered reodox state in hyporesponsive of T cells in rheumatoid arthritis. J. Immunol., **158**: 1458-1465.

Maurice M.M., Vander Voort E.A.M, Leow A., Levarht N., Breedveld F.C., and Verweij C.L., (1998) CD28 co-stimulation is intact and contributes to prolonged ex vivo survival of hyporesponsive synovial fluid T cells in rheumatoid arthritis. Eur. J. Immunol., **28**: 1554-1562.

Mauviel A., (1993). Cytokine regulation of metalloproteinase gene expression. J. Cellu. Biochem., **53**: 288-295.

McCarty D., 11th Edition. (1989). Arthritis and allied conditions. Lea & Febiger

McGuirk P., and Mills K.H.G. (2002). Pathogen-specific regulatory T cells provoke a shift in the Th1/Th2 paradigm in immunity to infectious diseases. Trends Immunol., **23**: 450-455

Mclnnes I.B., Gracie J. A., Leung B.P., Wei X.Q., and Liew F., (2000). Interleukin-18: pleiotropic participant in chronic inflammation. Immunol. Today, **21**: 312-315.

Merino F., Alvarezmon, M., Delahera A., Ales J.E., Bonilla F., and Durantez A., (1989). Regulation of natural-killer cyto-toxicity by 1,25-dihyroxyvitamin D3. Cellu. Immunol., **118**: 328-336.

Miller D.K., Ayala J.M., Egger L.A., Raju S.M., Yamin T.T., Ding J-F., Gaffney E.P., Howard A.D., Palyha O.C., Rolando A.M., Salley J.P., Thornberry N., Weider J.R., Williams J.H., Chapman K.T., Jackson J., Kostura M. J., Limjuco G., Molineaux S.M., Mumford R.A., and Calaycay J.R., (1993). Purification and characterization of active human interleukin-1β-converting enzyme from THP-1 monocytes. J. Biol. Chem., **268**: 18062-18069.

Mino T., Sugiyama E., Taki H., Kuroda A., Yamashita N., Maryuma M., and Kobayashi M., (1998). Interleukin-1α and tumor necrosis factor–α synergistically stimulate prostaglandin E2-dependent production of interleukin-11 in rheumatoid synovial fibroblasts. Arthitis Rheum., **41**: 2004-2013.

Miossec P. and Berg W., (1997). Th1/Th2 cytokine balance in arthritis. Arthritis Rheum., **40**: 2105-2115.

Miossec P., (2003). Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy. Arthritis Rheum., **48**: 594-601.

Monick M.M., Samavati L., Butler N., Mohning M., Powers L., Yarovinsky T., Spitz D.R., and Hunninghake G.W., (2003). Intracellular Thiols contribute to Th2 function via positive role in IL-4 production. J. Immunol., **171**: 5107-5115.

Moreland L.W., Bucy R.P., Jackson B., Feldman J. and Koopman W.J., (1997). Long-term follow up (30 months) of rheumatoid arthritis patient treated with a chimeric anti-CD4 monoclonal antibody (CmAb) Arthritis Rheum., **36**: 129-134.

Mosmann T.R., Schumacher J.H., Street N.F., Budd R., Garra A., Fong T.A., Bond M.W., Moore K.W., Sher A., & Fiorentino D.F., (1991). Diversity of cytokine synthesis and function of Mouse $CD4^+$ T cells. Immunol. Rev., **123**: 209-229.

Mulherin D., FitzGerrald O., and Brenihan B., (1996). Synovial tissue macrophage populations and articular damage in rheumatoid arthritis. Arthtitis Rheum., **39**: 115-124.

Murata Y., Shimamura T., and Hamura J., (2002). The polarization of Th1/Th2 balance is dependent on intracellular thiol redox status of macrophages due to the distinctive cytokine production. Int. Immunol., **14**: 201-212.

Muzio M, Re F, Sironi, Polentarutti N, Minty A., Caput D., Ferrara P., Mantovani A., and Collotta F. (1994). Interleukin-13 induces the production of interleukin-1 receptor antagonist (IL-1ra) and the expression of the mRNA for the intracellular (keratinocyte) form of IL-1ra in human myelomonocytic cells. Blood, **83**: 1738-1743.

Naldini A., Pucci A., Carraro F., (2001). Hypoxia induces the expression and release of interleukin 1 receptor antagonist in mitogen-activated mononuclear cells. Cytokine, **13**: 334-341

Nanki T., Hayashida K., El-Gabalawy H., Suson S., Shi K., Girschick H.J., Yavuz S., and Lipsky P.E., (2000). Stromal cells-derived factor-1 CXC chemokine receptor 4 interactions play a central role in CD4+ T cell accumulation in rheumatoid arthritis synovium. J. Immunol, **165**: 6590-6598.

Nemoto O., Yamada H., Mukaida M., and Shimmei M., (1996). Stimulation of TIMP-1 production by oncostain M in human cartilage. Arthitis Rheum., **39**: 560-566

Neumann D., Kollewe C., Martin M., and Boraschi D. (2000). The membrane form of the type II IL-1 receptor accounts for inhibitory function. J. Immunol, **165**: 3350-3357.

Novick D., Kim S.H., Fantuzzi G., Reznikov L.L. Dinarello C.A., and Rubinstein M., (1999). Interleukin-18 binding protein: A novel modulator of the Th1 cytokine response. Immunity, **10**: 127-136.

Obeid L.M., Okazaki T., Karolak L.A., and Hannun Y.A., (1990). Transcriptional regulation of protein kinase C by 1,25 dihydroxyvitamin D3 in HL-60 cells. J. Biol. Chem., **265**: 2370-2374.

Ohmori Y., Smith M.F., and Hamilton T.A., (1996). IL-4 induced expression of the IL-1 receptor antagonist gene is mediated by STAT6. J. Biol. Chem., **157**: 2058-2065.

Okamoto H., Yamamura M., Morita Y., Harada S., Makino H., and Ota Z., (1997). The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. Arthritis Rheum., **40**: 1096-1105.

Ollier W.E.R. and MacGreger A., (1995). Genetic epidemiology of rheumatoid disease. Br. Med. Bull., **51**: 267-285.

Oresto A., and Michel F., (2003). CD28-mediated co-stimulation: A quantitative support for TCR signalling. Nature, **3**: 939-951
Orlando S., Polentarutti N., Mantovani A., (2000). Selectivity release of the type II decoy IL-1 receptor. Cytokine, **12**: 1001-1006.

Ortiz A.M., Laffon A., and Gonzalez Alvaro I., (2002). CD69 expression on lymphocytes and interleukin-15 levels in synovial fluid from different inflammatory arthopathies., Rheumatol. Int., **21**: 182-188.

Palmer D.G., (1995). The anatomy of the rheumatoid lesion. Br. Med. Bull., **51**: 286-295

Panayi G.S., Landchbury J.S and Kingsley G.H (1992). The importance of the T cells in initiating and maintaining the chronic synovitis of rheumatoid arthritis. Arthritis Rheum., **35**: 729-735.

Panayi G.S., (1999). Targeting of cells involved in the pathogenesis of rheumatoid arthritis. Rheum., **38**: 8-10.

Park C.S., Park W-R., Sugimoto N., Nakahira M., Ahn H-J., Hamaoka T., Ohta T., Kurimoto M., Fujiwara H., (2000). Differential effects of N-acetyl-L-cysteine on IL-2 vs IL-12-driven proliferation of a T cell clone: implication for distinct signalling pathways. Cytokine, **12**: 1419-1422.

Park C.T., Creasey A.A., and Wright S.D., (1997). Tissue factor pathway inhibitor blocks cellular effects of endotoxin by binding to endotoxin and interfering with transferring to CD14. Blood, **89**: 4268-4274.

Park, S. H., and Aust, A.E., (1998). Regulation of nitric oxide synthase induction by iron and glutathione in asbestos-treated human lung epithelial cells. Arch. Biochem. Biophys., **360**: 47-52.

Parmentier M, Hirani N, Rahman I, Donaldson W, MacNee W, and Antonicelli F., (2000). Regulation of lipopolysaccharide-mediated interleukin-1β release by N-acetylcysteine in THP-1 cells. Eur. Respir J., **16**: 933-939.

Parry S., Sebbag M., Feldmann M., and Brennan F., (2000). Contact with T cells modulates monocytes IL-10 production. J. Immunol, **158**: 3673- 3681.

Patil N.S., Pashine A., Belmares M.P., Liu W., Kaneshiro B., Radinowitz J., McConnell H., and Millins E.D., (2001). Rheumatoid arthritis (RA)- Associated HLA-DR alleles form less stable complexs with class II- associated invariant chain peptide than non-RA-assocaited HLA-DR alleles. J. Immunol., **167**: 7157-7168.

Pedron T., Girard R., and Chaby R., (1995). Variation of LPS-binding capacity epitope expression and shedding of membrane-bound CD14 during differentiation of human monocytes. J. Immunol., **155**: 1460-1471.

Pelassy C., Mary D., Aussel C., (1991). Diacylglycerol production in Jurkat T cells: differences between CD3, CD2 and PHA activation pathways. Cell Signal., **3**: 35-40

Perlmutter R.M., Levin S.D., Appleby M.W., Anderson S.J., and Alberolaila J., (1993). Regulation of lymphocyte function by protein-phosphorylation. Ann. Rev. Immunol., **11**: 451-499.

Perregaux D.G., Laliberte R., and Gabel C.A., (1996). Human monocyte interleukin-1β posttranslational processing. J. Biol. Chem., **271**: 29830-29838.

Perregaux D.G., and Gabel C.A., (1998). Human monocyte stimulus-coupled IL-1β posttranslational processing: modulation via monovalent cations. Am. J. Physiol., **275**: C1538-C1547.

Perregaux D.G., McNiff P., Laliberte R., Conklyn M., Gabel C.A., (2000). ATP acts an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. J. Immunol., **165**: 4615-4623.

Peterson J., Herzenberg L., Vasquez K., and Waltenbaugh C., (1998). Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns. Proc. Natl. Acad. Sci., **95**: 3071-3076.

Pfeiffer A., Bottcher A., Orso E., Kapinsky M., Nagy P., Bodnar A., Spreitzer I., Liebisch G., Drobnik W., Gempel K., Horn M., Holmer S., Hartung T., Multhoff G., Schutz G., Schindler H., Ulmer A.J., Heine H., Stelter F, Schutt C., Roth G., Szollosi J., Damjanovish S., and Schmitz G., (2001). Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptors clustering in rafts. Eur. J. Immunol., **31**: 3135-3164.

Plater-Zyberk C., Joonsten L., Helsen M., Sattonnet-Roche P., Siegfried C., Alouani S., van de Loo F., Graber P., Aloni S., Cirillo R., Lubberts E., Dinarello C., van den Pugin J., Heumann D., Tomasz A., Kravchenko V., Akamatsu Y, Nishijma M., Glauster M, Tobias P. and Ulevitch R., (1994). CD14 is pattern recognition receptors. Immunity, **1**: 509-516.

Pugin J., Kravchenko V., Akamatsu M., Nishijma M., Glauster P., Tobias P. and Ulevitch R., (1998). Cell activation mediated by glycosylphosphatidylinositol anchored or transmembrane forms of CD14. Infec. Immun., **66**: 1174-1180.

Puren A., Fantuzzi, and Dinarello; (1999). Gene expression synthesis and secretion of interleukin-18 and interleukin-1β are differentially regulated in human blood mononuclear cells and mouse spleen cells. Proc. Natl. Acad. Sci., **96**: 2256-2261.

Putz E.F., and Mannel D.N., (1996). A role for L-selectin in monocytes activation by Jurkat tumour cells. Scand. J. Immunol, **44**: 556-564.

Ramirez R., Carracedo J., Castedo M., Zamzami N., and Kroemer G., (1996). CD69-induced monocyte apoptosis envolves multiple nonredundant signalling pathways. Cellu.Immunol., **172**: 192-199.

Ranges G.E., Figari I.S, Espevik T. and Palladino M.A., (1987). Inhibition of cytotoxic T cells development by transforming growth factor-beta and reversal by recombinant tumor necrosis factor-alpha. J. Exp. Med., **166**: 991-998.

Ranheim E.A., and Kipps T.J., (1994). Elevated expression of CD80 (B7/BB1) and other accessory molecules on synovial-fluid mononuclear cells subsets in rheumatoid arthritis. Arthitis Rheum., **37**: 1637-1646.

Reed D.J., (1986). Regulation of reductive processes by glutathione. Biochem. Pharmacol., **35**: 7-13.

Reid C.D., Stackpoole A., Maeger A., and Tikepae J., (1992). Interaction of tumor necrosis factor with granulocyte-macrophage colony stimulating factor and other cytokines in regulation of dendritic cell growth from early bipotent $CD34^+$ progenitors in human bone marrow. J. Immunol., **149**: 2681-2688.

Reiter T.A., Abraham R.T., Choi M., Rusnak F., (1999). Redox regulation of calcineurin in T lymphocytes. J. Biol. Inorg. Chem., **4**: 632-644.

Rezzonico R., Burger D., and Dayer J.M., (1998). Direct contact between T lymphocytes and human dermal fibroblasts or synoviocytes down-regulates types I and III collagen production via cell-associated cytokines. J. Biol. Chem., **273**: 18720-18728.

Rigby W.F.C., Stacy T., and Fanger M.W., (1984). Inhibition of lymphocyte-T mitogenesis by 1,25 dihydroxyvitamin–D3 (Calcitriol). J. Clin. Invest., **74**: 1451-1455.

Rincon M., Anguita J., Nakamura T., Fikrig E., and Flavell R.A., (1997). Interleukin-6 directs the differentiation of interleukin-4 producing cells $CD4^+$ T cells. J. Exp. Med., **185**: 461-469.

Rincon M., Flavell R.A., and Davis R., (2000). The JNK and p38 MAP kinase signalling pathway in T cell-mediated immune responses. Free Rad. Bio. Med., **28**: 1328-1337.

Romas E., Udagawa N., Zhou H., Tamura T., Saito M., Taga T, (1996). The role of the gp130-mediated signals in oseoclast development regulation of interleukin 11 production by oseoblasts and distribution of its receptor in bone marrow cultures. J. Exp. Med. **183**: 2581-2591.

Ross T.K, Darwish H.M and Deluca H.F., (1994). Molecular-Biology of Vitamin D_3 action. Vitamins and Hormones-Advances in research and application, **49**: 281-326

Roth L., Goebeler M., Vandenbos C., and Sorg C., (1993). Expression of calcium-binding protein MRP8 and MRP14 are associated with distinct monocytes differentiation pathways in HL-60 cells. Biochem. Biophy. Res. Commu. **191**: 565-570.

Roveri A., Coassin M., Maiorino M., Zamburlini A., van Amsterdam F.T., Ratti E., Ursini F., (1992). Effect of hydrogen peroxide on calcium homeostasis in smooth muscle cells. Arch. Biochem. Biophys., **297**: 265-270.

Ruth J.H., Rottman J.B., Katschke K.J., Qin S., Wu L., LaRosa G., Ponath P., Pope R.M., Koch A.E., (2001). Selective lymphocyte chemokine receptors expression in the rheumatoid joint. Arthritis Rheum., **44**: 2750-2760.

Sakkas L., Johanson N., Scanzello C., and Platsoucas C., (1998). Interleukin–12 is expressed by infiltrating macrophages and synovial lining cells in rheumatoid arthritis and osteoarthritis. Cellu. Immunol., **188**: 105 –110.

Sallusto F., Lanzavecchia A., and Mackay C.R, (1998). Chemokines and Chemokine receptors in T-cells priming and Th1/Th2 mediated reponses. Immunol Today, **19**: 568-574.

Salojin, K.V., Zhang J., Madrenas J.and Delovitch T.L. (1998). T cell energy and altered T cell receptor signaling effect on autoimmune diseases. Immunol. Today, **19**: 468-473.

Sampaio E.P., Oliveira R.B., Warwick-Davies J., Neto RB., Griffin G.E. Shattock R.J., (2000). T cell-monocyte contact enhance tumor necrosis-factor production in response to Mycobacterium leprae. J. Infect. Dis., 182: **1463**-1472.

Schett G., Tohidast-Akrad, Steiner G., and Smolen J., (2001). The stressed synovium. Arthritis Res., **3**: 80-86.

Schindler R., Gelfand J.A., and Dinarello C.A., (1990). Recombinant C5a stimulates transcription rather than translation of interleukin-1 (IL-1) and tumor necrosis factor: translation signal provided by lipopolysaccharide or IL-1 itself. Blood, **76**: 1631, abstract.

Schumann R.R., Lamping N., Hoess A., Yu B., Park T., Kirschning C., Pfeil D., Reuter D., Wright S.D and Herrmann F., (1996). Functional characterization of the lipopolysaccharide (LPS) binding site of LPS binding protein (LBP) by site-directed mutagensis. Br. J. Haem, **93**:1172. (abstract).

Schumann R.R., Belka C., Reuter D., Lamping N., Kirschning C., Weber J.R., and Pfeil D., (1998). Lipopolysaccharide activates caspase-1 (interleukin-1- converting enzyme) in culture monocytes and endothelial cells. Blood, **91**: 577-584.

Scott D.L., (2000). Prognostic factors in early rheumatoid arthritis. Rheumatol., **39**: 24-29.

Seckinger P., Isaaz S., and Dayer J-M., (1989). Purification and biologic characterization of a specific tumour necrosis factor α inhibitor. J. Biol. Chem., **264**: 11966-11973.

Seder R.A., and Paul W.E., (1994). Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. Ann. Rev. Immunol., **12**: 635-673.

Seitz M., Loetscher P., Dewald B., Towbin H., Rordorf C., Gallati H., Baggiolini M., and Gerber N.J., (1995). Methotrexate action in rheumatoid arthritis: stimulation of cytokine production by peripheral blood mononuclear cells. Br. J. Rheumtol., **34**: 602-609.

Seres T., Knickelbein R. G., Warshaw J.B., and Johnston R.B., (2000). The phagocytosis-associated respiratory burst in human monocytes is associated with increased uptake of glutathione. J. Immunol. **165**: 3333-3340.

Sewell K.L., and Trentham D.E., (1993). Pathogenesis of rheumatoid arthritis. Lancet, **34**: 283-286.

Shalom-Barak T., Jacquenline Q., and Lotz M., (1998). Interleukin-17 induced gene expression in articular chondrocytes is associated with activation of mitogen-activated protein kinases and NF-κB. J. Biol. Chem., **273**: 2767-27473.

Sharkey N.A., and Blumberg P.M, (1985). Highly lipophilic phorbol esters as inhibitors of specific [H-3] phorbol 12, 13-dibutyrate binding. Cancer Res., **45**:19-24.

Shin H. C., Benbernou N., Esnault Guenounou M., (1998). Expression of IL-17 in human memory $CD45RO^+$ T lymphocytes and its regulation by protein kinase A pathway. Cytokine; **11**: 257-266

Shrieve D.C., Bump§ E. A., and Rice G.C., (1988). Heterogeneity of cellular glutathione among cells derived from a murine fibrosarcoma or human renal cells carcinoma detected by flow cytometric analysis. J. Biol. Chem., **263**:14107-14114.

Simon L.S., and Yocum D., (2000). New and future drug therapies for rheumatoid arthritis. Rheumatol., **39**:36-42.

Sims J.E., Giri J.G., and Dower S.K., (1994). Two IL-1 receptors play different roles in IL-1 actions. Clin. Immunol. Immunopath., **72**: 9-14.

Singeal D. P., Li J. and Lei K., (1999). Genetics of rheumatoid arthritis (RA): two separate regions in the major histocompatibilty complex contribute to susceptibility to RA. Immunol. Lett., **69**: 301-306

Smith J.B., and Haynes M.K., (2002). Rheumatoid arthritis-A molecular understanding. Ann. Intern. Med., **136**: 908-922

Smith M.F., Eidlen D., Arend W.P., and Gutierrez-Hartmann A., (1994). LPS-induced expression of the human IL-1 receptor antagonist gene is controlled by multiple interacting promoter elements. J. Immunol., **153**: 3584-3593.

Smith M.F., Jr, Carl V.S., Lodie T., and Fenton M.J., (1998). Secretory interleukin-1 receptor antagonist gene expression requires both PU.1 and a novel composite NF-κB/PU.1/GA-binding protein binding site. J. Biol. Chem., **273**: 24272-24279.

Smith S., Brown-Galatola, and Hall N.D., (1992). Modulation of Human T cells functions by surface sulphydryl groups: differential effects on IL-2 production and responsiveness. Clin. Exp. Immunol., **88**: 169-173.

Snowden N., and Kay R.A., (1995). Immunology of systemic rheumatoid disease. Br. Med. Bull., **51**: 437-448.

Sorrentino V., and Rizzuto R., (2001). Molecular genetics of Ca^{2+} stores and intracellular Ca^{2+} signalling. Trends Pharmaco. Sci., **22**: 459-464.

Staal F.J.T., Anderson M.T., Staal G.E.J., Herzenberg L.A., and Gitler C., (1994). Redox regulation of signal–transduction–tyrosine phosphorylation and calcium influx. Proc. Natl. Acad. Sci., USA, **91**: 3619-3622.

Steiner G., Tohidast-Akrad M., Witzmann G., Vesely M., Studnicka-Benke A., Gal A., Kunaver M., Zenz P., and Smolen J., (1999). Cytokine production by synovial T cells in rheumatoid arthritis. Rheumatol., **38**: 202-213.

Stevens C.R., Williams R.B., Farrell D.R., and Blake D.R., (1991). Hypoxia and inflammatory synovitis: Observation and speculation. Ann. Rheum. Dis., **50**: 124-132.

Stoeck M., Warncke U., Brennecke M, and Resch K., (1987). Induction of responsiveness to interleukin-2 in mouse lymphocytes by synergistic action of ionophore A23187 and Diacylglycerol. Scand. J. Immunol., **26**: 85-89.

Striz I., Slavcev A., Kalanin J., Jaresova M., Rennard SI., (2001). Cell-cell contact with epithelial cells modulates the phenotype of human macrophages. Inflammation, **25**: 241-246.

Stylianou E., O'Neill, L.A.J., Rawlinson L., Edbrooke M.R., Woo P., and Saklatvala J., (1992) Interleukin-1 induces NF-kappa B through its typ-I but not type-II receptor in lymphocytes. J. Biol. Chem., **267**: 15836-15841.

Summers K.L., O'Donnell J.L., and Hart D.N., (1994). Co-expression of the CD45RA and CD45RO antigens on T lymphocytes in chronic athritis. Clin. Exp. Immunol., **97**: 39-44.

Suthanthiran M., Anderson M., Sharma V., and Meister A., (1990). Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens. Proc. Natl. Acad. Sci. USA, **87**: 3343-3347

Sutton B.J., and Cloud H.J., (1993). The human IgE network. Nature, **366**: 421-428.
Suzuki H., Takemura H., and Kashiwagi H., (1995). IL-1 receptor antagonist (IL-1 ra) in patients with systemic lupus erythematosus (SLE). FASEB J., **9**: A1029.

Swain S., Croft M., Haynes L., and Zhang X.H., (1994). Generation of Th1 and Th2 memory. FABES J., **8**: A1002.

Syrbe U., Siveke J., and Hamann A. (1999). Th1/Th2 subsets: Distinct differences in homing and chemokine receptor expression? Springer. Semin. Immunopathol.. **21**: 263 (Abstract).

Tacchini L., Pogliaghi G., Radice L., Anzon E., and Bernellizazzera A., (1995). Differential activation of heat-shock protein and oxidation specific stress genes in chemically induced oxidative stress. Biochem. J., **309**: 453-459.

Tak P., Smeets T., Daha M., Kluin P., Meijers K., Brand R., Meinders A. and Breedveld F., (1997). Analysis of the synovial cells infiltrate in early rheumatoid synovial tissue in relation to local disease activity. Arthritis Rheum., **40**: 217-225.

Tanaka C., Kamata H., Takshita H., Yagisawa H., and Hirata H., (1997). Redox regulation of lipopolysaccharide (LPS)-induced interleukin-8 (IL-8) gene expression mediated by NF-κB and AP-1 in human astrocytoma U373 cells. Biochem. Biophy. Res. Commu., **232**: 568-573.

Tarkowaski A., Klareskog L., Carlsten H., Herberts P. and Koopman W.J., (1989). Secretion of antibodies to type-I and II collegen by synovial tissue-cells in patient with rheumatoid arthritis. Arthritis Rheum., **32**: 1087-1092.

Taylor P.C., (2003). Antibody therapy for rheumatoid arthritis. Curr. Opin. Pharmacol., **3**: 323-328.

Testi R., D'Ambrosio D., De Maria R, and Santoni A., (1994). The CD69 receptor: a multipurpose cell-surface trigger for hematopoietic cells. Immunol. Today, **15**: 479-483.

Thannislass J., Raveendran M., and Devaraj H., (1995). Buthionine sulfoximine-induced glutathione depletion its effect on antioxidants, lipid peroxidation and calcium homeostasis in the lung. Biochem. Pharmacol., **50**: 229-234.

Thiele K., Reimann D., Navarrette S., Langner J., Kehlen A., (2000). Cell-cell contact of human T cells with fibroblasts changes lymphocytic mRNA expression: increased mRNA expression of interleukin-17 and interleukin-17 receptor. Euro. Cytokine. Netw., **11**: 53-58.

Thomas R., and Carroll G.J., (1993). Reduction of leukocyte and interleukin-1β concentration in the synovial fluid of rheumatoid arthritis patients treated with methotrexate. Athritis Rheum., **36**: 1244-1252.

Thomas R., and Quinn C., (1996). Functional differentiation of dendritic cells in rheumatoid arthritis: Role of CD86 in synovium. J. Immunol., **156**: 3074-3086.

Thornbury N., Bull H., Calaycay J., Chapman K., Howard A., Kostura M., Miller D., Molineaux S., Weidner J., Aunins J., Elliston K., Ayala J., Casano F., Chin J., Ding G., Egger L., Gaffney E., Limjuco G., Palyha O., Raju S., Rolando A., Salley J., Yamin T., Lee T., Shiverly J., MacCross M., Mumford R., Schmidt J,.and Tocci M., (1992). A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. Nature, **356**: 768-774.

Thurkow E.W., van der Heijden I.M., Breedveld F.C., Smeets T.J., Daha M.R., Kluin P.M., Meinders A.E., and Tak P.P, (1997). Increased expression of IL-15 in the synovium of patients with rheumatoid arthritis compared with patients with Yersinia-induced arthritis and osteoarthitis. J. Pathol., **181**:444-450.

Tilg H., Dinarello C.A., and Mier J.W., (1997). IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. Immunol. Today, **18**: 428-432.

Tobias PS., Soldau K., Kline L., Lee J.D., Kato K., Martin TP., Ulevitch R.J, (1993). Cross-linking of lipopolysaccharide (LPS) to CD14 on THP-1 cells mediated by LPS-binding protein. J. Immunol., **150**: 3011-3021.

Torigoe K., Ushio S., Okura T., Kobayashi S., Taniai M., Kunikata T., Murakami T., Sanou O., Kojima H., Fujii M., Ohta T., Ikeda H., and Kurimoto M., (1997). Purification and characterization of the human interleukin-18 receptor. J. Biol. Chem., **272**: 25737-25742.

Torsteinsdottir I., Arvidson N.G., Hallgren R., and Hakansson L., (1999). Monocyte activation in rheumatoid arthritis (RA): Increased intigrin, Fc gamma and complement receptors expression and the effect of glucocorticoids. Clin. Exp. Immunol., **115**: 554-560.

Toullec D., Pianetti P., Coste H., Bellevergue P., Grand-Perret T., Ajakane M., Baudet V., Boissin P., Boursier E., Loriolle F., Duhamel L., Charon D., and Kirilovsky J., (1991). The bisindolylmaleimide GF109203X is a potent and selective inhibitor of protein kinase C. J.Biol. Chem., **266**: 15771-15781.

Triantafilou M, and Triantafilou K, (2002). Lipopolysaccharide recognition: CD14, TLRs and the LPS-activation cluster. Trends Immunol., **23**: 301-304.

Trunech A., Albert F., Golstein P. and Schmittverhulst A.M., (1985). Calcium ionophore plus phorbol ester can substitute for antigen in the induction of cytolytic lymphocytes T from specifically primed precursors. J. Immunol., **135**: 2262-2267.

Tsitsikov E.N., Fuleihan R., Mcintosh K., Scholl P.R., and Geha R.S., (1995). Cross-linking of Fc-gamma receptors activates HIV-1 long terminal repeat-deriven transcription in human monocytes. Int. Immunol., **7**: 1665-1670.

Ublacker G. A., Johnson J. A., Siegel F., and Mulcahy T. (1991). Influence of glutathione S-transferases on cellular glutathione determination by flow cytometry using monochlorobimane. Cancer Res., **51**: 1783-1788.

Ulevitch R.J., and Tobias P.S., (1995). Receptor-dependent mechanism of cell stimulation by bacterial endotoxin. Ann. Rev. Immunol., **13**: 437-457.

Ulmer A.J., Flad H.D., Rietschel T., Mattern T., (2000). Induction of proliferation and cytokine production in the human T lymphocytes by lipopolysaccharide (LPS). Toxicology, **152**: 37-45.

Utsugi M, Dobashi K., Koga Y., Shimizu Y., Iizuka K., Hamura J., Nakazawa T., and Mori M., (2002). Glutathione redox regulates lipopolysaccharide-induced IL-12 production through p38 mitogen-activated protein kinase activation in human monocytes: role of glutathione redox in IFN-gamma priming of IL-12 production. J. Leukoc. Biol., **71**: 339-347.

Van de Loo F.A.J., Joosten L.A.B., Van Lent P. L.E., Arntz O.J., and Berg W. B. (1995). Role of interleukin-1, Tumour necrosis factor-α, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Arthritis Rheum., **38**: 164-172.

Van de Winkle J.G.J., and Anderson C.L.(1991). Biology of human immunoglobulin-G Fc receptors. J. Leukoc. Biol., **49**: 511-524.

Van der Ven A.J., Mier P., Peters W.H., Dolstra H., van Erp P.E.J., Koopmans P.P., and van der Meer J.W., (1994). Monochlorobimane does not selectively label glutathione in peripheral blood mononuclear cells. Anal. Biochem., **217**: 41-47.

Venables P.J. (1989). Infection and rheumatoid arthritis. Curr. Opin. Rheumatol., **1**: 15-20.

Verhasselt V., Berghe W.H., Vanderheyde N., Willems F., Haegeman G., and Goldman M., (1999). N-acetyl-L-cysteine inhibits primary human T cell responses at the dendritic cell level: Assocaition with NF-κB inhibition. J. Immunol., **162**: 2569-2574.

Vervoordeldonk M.J., and Tak P.P., (2002). Cytokines in Rheumatoid arthritis, Curr. Rheumatol. Rep.., **4**: 208-217.

Vey E., Zhang J.H. and Dayer J-M. (1992). IFN-gamma and 1,25 (OH) $_2$ D3 induce on THP-1 cells distinct pattern of cell-surface antigen expression, cytokine production and responsiveness to contact with activated T cells. J. Immunol, **149**: 2040-2046.

Vey E., Dayer J-M., and Burger D., (1997). Direct contact with stimulated T cells induces the expression of IL-1β and IL-1 receptor antagonist in human monocytes. Involvement of serine/threonine phosphotases in differential regulation. Cytokine, **9**: 480-487.

Vincenti M.P., Coon C.I., Lee O., and Brinckerhoff C.F., (1994). Regulation of collagenase gene expression by IL-1-beta requires transcriptional and posttranscriptional mechanisms. Nuc. Acids Res., **22**: 4818-4827.

Vincenti M.P., and Brinckerhoff C.F., (2001). The potential of signal transduction inhibitors for the treatment of arthritis: is it all just JNK? J. Clin. Invest., **108**: 181-183.

Vincenti M.P., and Brinckerhoff C.F., (2002). Transcriptional regulation of collagenase (MMP-1, MMP-8 and MMP13) gene in arthritis. Arthitis Res, **4**: 157-164.

Viora M., Quaranna M., Straface E., Vari R., Masella R., and Malorni W., (2001). Redox imbalance and immune functions: opposite effect of oxidized low-density lipoproteins and N-acetlycysteine. Immunol., **104**: 431-438.

Vreugdenhill A.C., Dentener M., Snoek A.M., P., Greve J-W., and Burman W.A., (1999). Lipopolysaccharide Binding protein and serum amyloid A secretion by human intestinal epithelial cells during the acute phase response. J. Immunol., **163**: 2792-2798.

Wagner D.H., Stout Jr.R.D., and Suttles J., (1994). Role of CD40-CD40 ligand interaction in monocytes interleukin-1 synthesis. Eur. J. Immunol., **24**: 3148-3154.

Walev I., Weller U., Strauch S., Foster T., and Bhakdi S., (1996) Selective killing of human monocytes and cytokine release provoked by sphingomylinase (beta-toxin) of staphylococcus aureus. Infect. Immunol., **64**: 2974-2979.

Wang H., and Joseph J.A., (2000). Mechansim of hydrogen peroxide-induced calcium deregulation in PC12 cells. Free Rad. Biol. Med., **28**: 1222-1231.

Wang P.Y., Kitchens R.L., and Munford R.S., (1998). Phosphatidylinositides binds to plasma membrane CD14 and can prevent monocytes activation by bacterial lipopolysaccharide. J. Biol.Chem., **273**: 24309-24313.

Ward N.E., Pierce D.S., Chung S.E., Gravitt K.R., O'Brain C.A., (1998). Irreversible inactivation of protein kinase C by glutathione. J. Biol. Chem., **273**:12558-12566.

Weinstein S.L, Sanghera J.S., Lemke K., De Franco A.L. and Pelech S.L. (1992). Bacterial Lipopolysaccharide induces tyrosin phosphorylation and activation of mitogen activated protein-kinases in macrophages. J.Bio.Chem., **267**: 14955-14962.

Weinstein S.L, June C.H., and De Franco A.L. (1993). Lipopolysaccharide induced protein tyrosin phosphorylation in human macrophages is mediated by CD14. J. Immunol., **151**: 3829-3838.

Weiss M.J., Daley J.F., Hodgdon J.C., and Reinherz E.L., (1984). Calcium dependency of antigen-specific (T3-Ti) and alternative (T11) pathways of human T cell activation. Proc. Natl. Acad. Sci, USA, **81**: 6836-6840.

Weyand C.M (2000). New insight into the pathogenesis of rheumatoid arthritis. Rheumatol., **39**:3-8.

Whisler R.L., Goyette M.A., Grants I. S., and Newhouse Y.G., (1995). Sublethal levels of oxidant stress stimulate multiple serine/threonine Kinases and suppress protein phosphatases in Jurkate cells. Arch. Biochem. Biophy. **319**: 23-35.

Wijngarden S., van Roon J.A., Bijlsma J.W., van de Winkel and Lafeber F.P., (2003). Fcγ receptor expression levels on monocytes are elevated in rheumatoid arthritis patients with high erythrocyte sedimentation rate who do not use anti-rheumatic drugs. Rheumatol., **42**: 681-688.

William R., Watson G., Rotstein O.D, Parado J., Bitar R., and Marshall J.C., (1998). The IL-1β-Converting enzyme (caspase-1) inhibits apoptosis of inflammatory neutrophils through activation of IL-1β. J. Immunol, **161**: 957-962.

Wilson L., Butcher C.J., and Kellie S., (1993). Calcium ionophore A23187 induces interleukin-8 gene-expression and protein secretion in human monocytic cells. FEBS Letters **325**: 295-298.

Winchester R., Gregersen P.K., Merryman P., Dwyer E., Lee S., (1988). Hypotheses on the molecular basis of susceptibility to rheumatoid arthritis. Scand. J. Rheumatol. Suppl., **76**: 9-16.

Wong W.M; Vakis S.A.; Ayre K.R., Ellwood C.N., Howell W.M.; Tutt A.L., Cawley M.I.D.; Smith J.L., (2000). Rheumatoid arthritis T cells produce Th1 cytokines in response to stimulation with a novel trispecific antibody directed against CD2, CD3 and CD28. Scand. J. Rheumatol., **29**: 282-287.

Woods J., Katschke, Tokuhira M., Kurata H., Arai K., Campbell P., and Koch A., (2000). Reduction of inflammatory cytokine and prostaglandin E2 by IL-13 gene therapy in rheumatoid arthritis synovium. J. Immunol, **165**: 2755-2763.

Wooten R.M., Morrison T.B., Weis J.H., Wright S. D., Thieringer R., Weiss J.J, (1998). The role of CD14 in signalling mediated by outer membrane lipoproteins of *Borrelia burgdorferi*. J. Immunol, **160**: 5485-5492.

Wordsworth B.P., Lanchbury J.S.S, Sakkas L.I., (1989). HLA-DR4 subtype frequencies in the rheumatoid arthritis indicates that DRB1 is the major susceptibility locus within the HLA class II region. Proc. Natl. Acad. Sci., USA, **86**: 10049-10053.

Wright J.K, Cawston T.E., and Hazleman B.L., (1991). Transforming growth factor-beta stimulates the production of the tissue inhibitor of metalloproteinase (TIMP) by human synovial and skin fibroblast. Biochimica et Biophyysica Acta, **1094**: 207-210.

Wright S.D., Ramos R.A., Tobias P.S., Ulevitch R.J., Mathison J.C., (1990). CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding-protein. Science, **249**: 1431-1433.

Wurfel M.M., Lichenstein H., and Wrieght S.D., (1994). Binding and neutralizating of lipopolysaccharide by lipoproteins is blocked by soluble CD14 and LBP. FABES J., **8**: A316.

Xu H., Heeger P.S., and Fairchild R.L., (1997). Distinct roles for B7-1 and B7-2 determinats during priming effector CD8+ Tc1 and regulatory CD4+ Th2 cells for contact hypersensitivity. J. Immunol., **159**: 4217-4226.

Xu K.Y., Zweier J.L., Becker L.C., (1997). Hydroxyl radical inhibits sarcoplasmic reticulum $Ca^{(2+)}$-ATPase function by direct attack on the ATP binding site. Circ. Res., **80**: 76-81.

Yao J., Mackman N., Edgington T.S., and Fan S-T., (1997). Lipopolysaccharide induction of the tumor necrosis factor-α promotor in human monocytic cells. J. Biol. Chem., **272**: 17795-17801.

Yao Z., Painter S., Franslow W., Ulrich D., Macduff B., Springgs M. K., Armitage R., (1995). Human IL-17: A novel cytokine derived from T cells. J. Immunol., **155**: 5483-5486.

Ye K., Dinarello C.A., and Clark B.D., (1993) Identification of the promotor region of human interleukin-1-type-1 receptor gene-multiple initiation sites, high G+C content, and constitutive expression. Proc. Natl. Acad. Sci. USA, **90**: 2295- 2299.

Yellin MJ, Brett J, Baum D, Matsushima A, Szabolcs M, Stern D., Chess L., (1995). Functional interaction of T cells in rheumatoid arthritis. J. Clin Invest., **100**: 2404-2414

Yoshida S., Katoh T., Tetsuka T., Uno K., Matsui N., and Okamoto T. (1999). Involvement of thioredoxin in rheumatoid arthritis: Its costimulatory roles in the TNF-α–induced production of IL-6 and IL-8 from cultured synovial fibroblasts. J. Immunol., **163**: 351-358.

Yoshida A., Takahashi H.K., Nishibori M., Iwagaki H., Yoshino T., Morichika T., Yokoyama M., Kondo E., Tadaatsu A., and Tanaka N., (2001). IL-18-induced expression of intracellular adhesion molecule-1 in human monocytes: Involvement in IL-12 and IFN-γ production in PBMC. Cell. Immunol., **210**: 106-115.

Zafarullah M., Li W.Q., Sylvester J., and Ahmad M (2003). Molecular mechanisms of N-acetylcysteine actions. Cell. Mol. Life. Sci., **60**: 6-20.

Zarember K.A. and Godowski P.J., (2002). Tissue expression of human Toll-Like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products and cytokines. J. Immunol., **168**: 554-561.

Zhang F.X., Kirschning C.J., Maninelli R., Xu X.P., Jin Y., Faure E., Mantovani A., Rothe M., Muzio M., and Arditi M., (1999). Bacterial lipopolysaccharide activates Nuclear Factor-κB through interleukin-1 signalling mediators in cultured human dermal endothelial cells and mononuclear phagocytes. J. Biol. Chem., **274**: 7611-7614.

Zhang G., and Ghosh S., (2001). Toll-like receptor-mediated NF-κB activation: a phylogentically conserved paradigm in innate immunity. J. Clin. Invest., **107**: 13-19.

Zvaifler N.J., (1995). Macrophages and the synovial lining. Scand. J. Rheumatol. Suppl., **101**: 67-75.